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CONSISTENCY AND REPRODUCIBILITY OF BIOAEROSOL DELIVERY FOR INFECTIVITY STUDIES ON MICE

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14. ABSTRACT

Questions about the clinical significance of an antimicrobial resin use don personal respirators led system to generate consistent test bioaerosols for use in animal studies. The hypothesis was proposed that an aerosol delivery system based on the Collision nebulizer can be designed and engineered to provide, at selectable concentrations, a respiratory challenge of bioaerosol particles that is verifiably consistent in time and that can be fed in separate experiments through treated and untreated filters to deliver a consistent challenge to a small-animal model of human respiration.

To verify this hypothesis, such an experimental filtration system was designed and built. Challenge trials were performed with MS2 bacteriophage and *Bacillus atrophaeus*. Over 30 to 40 minutes, the particle size distribution (PSD) was measured, and viability of microorganisms collected in All-Glass Impingers (AGI-4s) was determined. Concentrations of particles and microorganisms downstream of the filter were too low to measure, and the viable counts for MS2 bacteriophage were not measured at all owing to problems with the assay method. However, in each experiment, the coefficients of variations (CVs) of time-series measurements of the total particle count, count median diameter, and geometric experiments with viability data, all CVs of time-series measurements of upstream viable airborne concentration were less than 26%. This CV is somewhat higher than has been reported in the literature for tests with airborne *Bacillus* spores, but the plating method used to measure the viability may have introduced additional variation that was not caused within the system itself. It can be reasoned based on this data that the system can provide a sufficiently steady aerosol challenge to be used for later studies using a small-animal model of human respiration. The system provides a design for an animal exposure system incorporating aerosol filtration, which is a capability previously unreported in the literature.

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CONSISTENCY AND REPRODUCIBILITY OF BIOAEROSOL DELIVERY FOR INFECTIVITY STUDIES ON MICE

By

BRENTON R. STONE

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
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2010

To my family and friends

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TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AGI	All-Glass Impinger
APS	Aerodynamic particle sizer
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
BSL	Biosafety level
C	Concentration (typically of particles, with units #/m^3, or of viable microorganisms, with units PFU/m^3 or CFU/m^3)
C_{down}	Concentration upstream downstream of a filter
C_{up}	Concentration upstream of a filter
cm	Centimeter
CATS	Controlled Aerosol Test System
CFU	Colony-forming unit
CMD	Count median diameter
CV	Coefficient of variation
DI	Deionized
EPA	(United States) Environmental Protection Agency
Δp	Pressure drop
F	Fractional deposition
FFR	Filtering facepiece respirator
GSD	Geometric standard deviation
HEPA	High efficiency particulate air
HVAC	Heating, ventilation, and air conditioning
in H_2O	Inches of water pressure
IPA	Isopropyl alcohol (2-propanol)
L	Liter

MERV	Minimum Efficiency Reporting Value
MID_{50}	Median infective dose (Minimum infective dose for 50% of the population)
mL	Milliliter, cubic centimeter
mm	Millimeter
MPPS	Most-penetrating particle size
µm	Micrometer
N	Count of PFUs or CFUs
n	Dilution factor
N95	A FFR with a PRE of 95% or greater for 300-nm salt particles
ND	No data
NIOSH	(United States) National Institute of Safety and Health
NIST	(United States) National Institute of Standards and Technology
nm	Nanometer
P95	An oil-resistant FFR with a PRE of 95% or greater for 300-nm salt particles
PBS	Phosphate-buffered saline
PFU	Plaque-forming unit
PRE	Physical removal efficiency
PSD	Particle size distribution
psi	Pounds per square inch pressure
psig	Pounds per square inch pressure over gauge pressure
PSL	Polystyrene latex
PSTI	poly(styrene-4-[trimethylammonium]methyl triiodide)
Q	Flow rate
Q_a	Flow rate of aerosol collected

R^2	Coefficient of determination for a linear regression
RH	Relative humidity
SARS	Severe acute respiratory syndrome
SMPS	Scanning mobility particle sizer
St. dev.	Standard deviation
T	Temperature
t	Time; duration of exposure; duration of sample
TPC	Total particle count
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
VEE	Venezuelan equine encephalomyelitis
V_i	Volume of liquid in impinger
V_m	Minute volume of an animal (units mL/min)
V_p	Volume of liquid plated
VRE	Viable removal efficiency
VSF	Viable spray factor

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Questions about the clinical significance of an antimicrobial resin used on personal respirators led to the need for a system to generate consistent test bioaerosols for use in animal studies. The hypothesis was proposed that an aerosol delivery system based on the Collison nebulizer can be designed and engineered to provide, at selectable concentrations, a respiratory challenge of bioaerosol particles that is verifiably consistent in time and that can be fed in separate experiments through treated and untreated control filters to deliver a consistent challenge to a small-animal model of human respiration.

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diameter, and geometric standard deviation of the upstream PSD were less than 10%. From five *B. atrophaeus* experiments with viability data, all CVs of time-series measurements of upstream viable airborne concentration were less than 26%. This CV is somewhat higher than has been reported in the literature for tests with airborne *Bacillus* spores, but the plating method used to measure the viability may have introduced additional variation that was not caused within the system itself. It can be reasoned based on this data that the system can provide a sufficiently steady aerosol challenge to be used for later studies using a small-animal model of human respiration. The system provides a design for an animal exposure system incorporating aerosol filtration, which is a capability previously unreported in the literature.

CHAPTER 1
INTRODUCTION

Motivation

Bioaerosols

An aerosol is any sort of finely divided material suspended in a gas. Bioaerosols are aerosols made up of particles of biological origin. Included in the category of bioaerosols are airborne viruses, bacteria, fungi, pollen, and organic material produced by biological processes (such as dust mite waste, a common household allergen).[1] Bioaerosols are known to be a transmission mechanism for many disease-causing organisms, including *Legionella*, smallpox, severe acute respiratory syndrome (SARS) coronavirus, and rhinovirus.[2] Whether bioaerosols are an important natural transmission mechanism for influenza is hotly debated.[3–10] Besides the huge impact of naturally occurring infectious disease in humanity, there is concern that infectious organisms could be weaponized in a bioaerosol form and used for biological warfare or terrorism. A great deal of research has gone into examining the weapon potential of bioaerosols and how to defend against potential threats.

A distinction is drawn between bioaerosols that are viable, or capable of being cultured, and those that are nonviable. A non-viable organism cannot infect a host. The viability of bioaerosols can change depending on a number of factors, including relative humidity (RH), temperature, oxygen content, airborne ions, radiation, and “open air factors” (a set of ambiguous influences that cause faster inactivation in outdoor air than in clean laboratory air).[11] Different organisms react differently to stresses in the aerosol state: the influenza virus is strongly affected by humidity,[12] and the stability of the bacterium *Escherichia coli* depends on RH, temperature, oxygen content, and the

aerosol generating method, while the bacteria *Bacillus subtilis* does not show a strong dependence on RH, temperature, or oxygen content.[11] For some microorganisms, an unfavorable humidity can decrease the airborne viable concentration by several orders of magnitude.[13] For viruses, the presence or absence of a lipid coating on the virions – a quality that varies by strain – alters whether the virus is more stable at high or low RH.[11] Among bacteria, the airborne viability and its dependence on RH and oxygen content differs greatly between Gram-negative and Gram-positive species.[13]

Bioaerosol particles vary in size depending on what microorganisms they contain. Individual virions tend to be 0.02 to 0.3 µm in physical diameter, bacteria 0.3 to 10 µm, fungal spores 0.5 to 30 µm, and pollen 10 to 100 µm. However, the individual particles may agglomerate into larger ones, or combine with non-biological airborne particles, increasing their dimensions and changing their behavior in the aerosol state.[14]

Creating Bioaerosols

Artificial bioaerosols are generated in the laboratory to simulate naturally occurring bioaerosols, for example to simulate a cough or an intentional release of an infectious agent as a biowarfare agent. The Collison nebulizer is often used to create a bioaerosol from a fluid containing microorganisms. In the Collison, a pressurized (typically 25 to 30 psig) stream of air draws up a liquid by the Venturi effect and jets it as a stream of droplets against the wall of its container. Of these droplets, those that are small enough are swept out of the nebulizer in the aerosol state; the rest return to the liquid reservoir.[15,16] Because Collison nebulizers are recirculating systems and impose large shear forces, microorganisms in suspension accumulate metabolic damage as a Collison continues to operate, and may lose viability.[17] The rate of loss of viability is typically not rapid enough to prevent an experiment from being performed. A quantity

used in evaluating the effectiveness of delivering a viable bioaerosol with nebulization is the viable spray factor (VSF). The VSF is defined as the ratio of the concentration in the aerosol state produced by the nebulizer to that in the nebulization liquid. On average, VSFs are of magnitude 10^{-7}, depending on the hardiness of the particular strain.[18]

Other methods of producing bioaerosols include other modes of atomization, such as ultrasonic nozzles that use high-frequency vibrations to produce an aerosol, and electrostatic nebulizers that use electrical forces. Dry powder dispersion techniques are used to produce an aerosol from a powder source, such as dry bacterial spores, and powder scrapers are also used for fungal bioaerosols.[16] These methods all have advantages, but are more complicated than a Collison, which has no moving parts.

Measuring Bioaerosols

Bioaerosols can, like any other aerosol, be measured with a particle sizer. Particle sizers measure the distribution of particles in a sample as a function of aerodynamic diameter. (The aerodynamic diameter of a particle is the equivalent diameter of a spherical particle of density 1 g/mL that has the same aerodynamic behavior, and is sometimes casually referred to as the "size.") From this particle size distribution (PSD), the particles in a certain size range can be counted, or moments of the PSD can be measured. The distribution can be measured in terms of particle count concentration, particle mass concentration, or a number of other ways. Aerosols – in particular aerosols produced by nebulizing a liquid or nebulizing a solution of dissolved solids and then drying the produced droplets – often follow a log-normal size distribution, and in those cases can be defined by a total particle concentration (TPC) (if mass-based, a total mass concentration), an average (for instance, count median diameter (CMD), mass mean diameter, etc.), and a geometric standard deviation (GSD) quantifying the

spread of the distribution.[19] If the nebulization liquid contains particles (such as microorganisms) larger than the mode for dissolved solids in a concentration sufficiently small that they do not agglomerate, the particle sizer detects them as a sharp peak near the diameter of the individual particles.[19] A number of particle sizers can be purchased, each model relying on different operating principles and having different capabilities.

Researchers also want to collect bioaerosols for later analysis. In practice, one of the most common methods is to collect bioaerosols in impingers. Impingers jet the bioaerosol into a liquid medium that traps a size-dependent fraction of the particles. Other methods include impacting onto a medium, and collecting onto filters that can be weighed to measure the mass of aerosol or dissolved to recover the sample.[20] No collection method is a perfect collector, and all collection methods impose some stress on the bioaerosol and cause a fraction of the collected microorganisms to become non-viable. For instance, Hogan et al.[21] measured the viable collection efficiency of all-glass impingers (AGIs) for particles of 30 to 100 nm diameter as being below 10%, increasing for larger and smaller particles. Grinshpun et al.[22] measured the bounce and reaerosolization from impingers: a significant quantity of particles escape from the impinger. Viable collection efficiency of impingers can depend on sampling time and RH.[23] The longer microorganisms are held in the collection medium before incubation, the more viability may be lost.[24] The efficiency and loss in a sampler may even depend on individual strains.[25] The degree of loss is difficult to quantify. However, animal experiments often compare dose-dependent responses to deduce the relative reduction in the dose. The values of these parameters do not need to be exactly known as much as they need to remain constant for the duration of timed-exposure experiments.

Microorganisms collected by these methods can be cultured to measure viability. Viable bacteria can be quantified by performing a plate assay and counting colony-forming units (CFUs); viruses, by performing a plate assay in a host microorganism and counting plaque-forming units (PFUs). Other methods exist to quantify bioaerosols. Assays can be performed for endotoxins specific to a organism, which is useful when the endotoxin causes disease.[26] A polymerase chain reaction assay can be performed to measure the amount of D- or RNA characteristic to the organism that appears in a sample: real-time versions of this method are in development specifically for bioaerosols.[27] Neither of these methods is sensitive to viability.

Filtration

Filtration by flowing an aerosol through a fibrous porous medium is a well-known and accepted method used in respiratory protection systems to remove unwanted particles, such as infectious bioaerosols, from breathing air. Filters require lower pressure drops (Δp) than other particle control systems and are efficient for a wide range of particle challenges, including very small particles and low particle concentrations. Filters are also relatively simple to use.[28] Other particle control systems, such as cyclones, electrostatic precipitators, or wet scrubbers, are typically large pieces of machinery that require industrial blowers, or require a large degree of upkeep.[28] In non-industrial situations, like a heating, ventilation, and air conditioning (HVAC) system in the home or office, or a personal respirator, filtration is a reasonable and common choice. The process of filtration relies on five basic mechanisms:[16]

Gravitational settling. Gravity draws the particle off its streamline and onto a surface, where it is captured. In filters, this mechanism is unimportant compared to

others unless the face velocity through the filter is extremely low or particles are extremely large.

Interception. A particle travelling along a streamline makes tangential contact with a filter fiber and is captured. The particles must have large enough dimensions to contact the filter fiber while remaining on the streamline.

Inertial impaction. Because of its momentum, a particle deviates from a streamline, makes contact with a filter fiber, and is captured. This is the dominant mechanism for particles with large inertia.

Diffusion. A particle travelling along a streamline experiences random deviations from its path due to Brownian motion. If these deviations cause the particle to contact a filter fiber, it is captured by diffusion. This is the dominant mechanism for small particles.

Electrostatic attraction. The electrostatic force on particles from charged filter fibers can move a particle off a streamline onto a surface. Many common filters use an electrically charged (“electret”) filter medium. The contribution of electrostatic attraction to filtration efficiency can be very large, but quantifying it requires knowing the charge on the particles and filter material at a microscopic level, which is difficult to measure.

The efficiency of a filter is typically quantified as the physical removal efficiency (PRE), which is the fraction of aerosol removed by the filter relative to the feed material. PRE is calculated as Equation 1-1, where C_{up} is the concentration of particles upstream and C_{down} is the concentration downstream. This efficiency can be at a specific size or for a range of particles. One can also quantify the viable removal efficiency (VRE), the fraction of viable particles removed by the filter relative to the feed. VRE is calculated the same as PRE, except with concentrations of viable microorganisms. For filters with

no special antimicrobial capability the VRE is close to the PRE at the particle size of the microorganism. All filters have a range of particle aerodynamic diameters for which the dominant process transitions from diffusion to impaction, and this range is the window of dimensions in which a given filter medium captures least efficiently. The PRE decreases with increasing face velocity, as does the most-penetrating particle size (MPPS).[29]

$$\mathrm{PRE} = 1 - \frac{C_{\mathrm{down}}}{C_{\mathrm{up}}} \tag{1-1}$$

Efficiency can be increased by increasing the thickness of the layers of the material, but Δ*p* across the media increases proportionally. The PRE and Δ*p* of a mechanical filter increase as more particles are loaded onto the filter.[28] However, excessive loading can damage a filter and reduce its PRE, and certain particles, such as dioctyl phthalate and NaCl, can reduce the electric charge on electret filters and thereby decrease their PRE. Barret and Rousseau[30] showed that this reduction in PRE can differ between filter media made from the same substance with different fiber production methods. In their article, some filters made from polypropylene show minimal change in Δ*p* while their PRE reduces, and some show a large increase in Δ*p*.

High Efficiency Particulate Air (HEPA) filters are used in building ventilation systems where biological isolation is desired. HEPA filters are defined to be 99.97% efficient at filtering 300-nm particles at a specified face velocity. Heimbuch et al.[31,32] showed that during challenges with practically attainable particle concentrations, biological pathogens with aerodynamic diameters in the range of 100 to 300 nm penetrate HEPA filters at the predicted fraction of 0.03%. The MS2 coli phage used in their test is not infective in humans, but a number of infectious microorganisms such as *Francisella tularensis*, whooping cough, SARS coronavirus, Venezuelan equine

encephalomyelitis, and the influenza virus could allegedly form particles in the 100- to 300-nm range by accumulating salts and organic matter on their surface. The median infectious dose (MID_{50}) for *F*. *tularensis* is 10 to 50 organisms, and while the MID_{50}s of the viruses are not known exactly, many are believed to be fewer than 10 organisms, possibly as few as a single organism for SARS.[33,34] These MID_{50}s are low enough that they could be surpassed by particles penetrating a HEPA filter.[32]

Filtration is also used for individual respiratory protection. A common piece of personal protective equipment for use with hazardous aerosols is the filtering facepiece respirator (FFR), a filter that covers a person's nose and mouth. The US National Institute for Occupational Safety and Health (NIOSH) approves FFRs in classes by PRE – classes 95, 99, and 100 denote filter media with at least 95%, 99%, or 99.97% PRE, respectively, at the most-penetrating particle size (MPPS) when tested with a NaCl aerosol – and by oil resistance: N, R, and P for non-resistant, somewhat resistant, and strongly resistant, respectively.[35] N95 and P95 respirators are the most commonly used, and have accordingly been studied extensively.[34,36,37] Without proper fit to a person's face, a respirator can leak around its seal, causing the level of protection provided to fall drastically below its PRE classification.[38] Even with proper fit, Bałazy and colleagues[39,40] showed that particles can penetrate a nominal N95 medium at a fraction greater than 5% (although they declined to name the specific models of FFR tested). The protection provided by the mask while worn is not necessarily equivalent to the filtration efficiency of the filter fabric on its own.

Another risk in the use of filters is fomites, which are inanimate objects capable of transmitting infectious organisms. Because infectious particles are trapped within the

fibers of a filter, the filter may become a fomite. The filter may protect a person from infection until he handles it and acquires an infectious organism by contact transmission.[41,42] The threat from fomites is severe enough that a National Academy of Science committee recommended that personal FFRs not be reused at all.[43]

Designing and choosing a respirator involves balancing the risk of infection with the suitability of the respirator for the specific work environment and user.[34] Creating a more mechanically efficient FFR is difficult because the Δp across the filter cannot be so high that breathing is uncomfortable while wearing the mask. Hence, simply adding more layers of fabric is often not an option.

Antimicrobials for Aerosol Filtration

Antimicrobials have been examined as a way to enhance air purification systems by killing microorganisms in addition to and in lieu of capturing them while minimally affecting the mechanical properties of the filter. Such filters are typically impregnated or coated with an antimicrobial substance, although the surface chemistry of fibers can also be modified.[44] Generally antimicrobials serve to chemically cause metabolic or structural damage to the microorganism and cause it to become non-viable: the mechanism differs for different antimicrobials, and can vary with the presence or absence of other substances, such as water vapor in an airstream.[45]

Marchin et al.,[46] in work on water disinfectants, draw a distinction among antimicrobials that are constant-release, which release a background of antimicrobial into the fluid; demand-release, which release antimicrobial only in the presence of microorganisms; and contact, which require the microorganisms to make physical contact with the antimicrobial. In the aerosol state, contact antimicrobials would require some form of capture, bounce, or reaerosolization to occur: while this would help

prevent the filter from becoming a fomite, it would minimally affect an aerosol passing through the filter. The common test in which a microorganism is streaked across an antimicrobial fabric in a Petri dish does not tell the whole story of the antimicrobial's mechanism when used in an aerosol filter.

Common antimicrobials used in aerosol filtration include quaternary ammonium compounds,[47] *N*-halamines, iodine compounds, and silver.[45,48–50] Foarde et al.[45] studied what were, at the time, the three antimicrobials registered with the US Environmental Protection Agency (EPA) for use on aerosol filters. They found those antimicrobials to be effective and noted that the application of the antimicrobials did not appreciably affect the filter's PRE. Verdenelli et al.[48] showed that if fiberglass filters were loaded with bacteria or fungi, the filters treated with quaternary amines had (depending on microorganism) much lower or zero counts of viable microorganisms, which prevented their becoming fomites. The same group studied a range of antimicrobials on fiberglass filters.[49] They found that not all antimicrobial substances they examined, including a formulation of quaternary amines, are chemically compatible with the fiberglass material. For the bacteria and fungi they tested, a different quaternary amine was more successful than the others. However, Marchin et al.[46] state that in water quaternary amines are not effective against viruses and cysts.

Antimicrobial silver is beginning to fall out of favor because of overuse and subsequent evolving bacterial resistance.[51] Also, silver is not effective against viruses in water and suspected to be ineffective in air as well.[46] Sullivan found minimal antimicrobial capacity in a commercial air filter containing silver.[50] Some researchers are still examining its use.[52] In more-recent work silver is examined in combination with

other antimicrobials, such as titanium dioxide.[53] Other metals have some antimicrobial action, but data describing their effectiveness in the aerosol state are rare. Byeon et al.[54] suggest that copper-coated activated carbon could be used as an antimicrobial and also for its adsorptive capabilities, although they do not use an aerosol challenge.

While a good deal of literature on aqueous antimicrobials exists, information on antimicrobials for aerosol filtration is surprisingly sparse, especially considering the commercial availability of antimicrobial aerosol filters. Sometimes researchers assume that the mechanisms in water and air are the same, but this is not always an easy or valid assumption to make, as shown in the next section.

The Antimicrobial Poly(styrene-4-[trimethylammonium]methyl triiodide)

Safe Life Corporation (San Diego, CA, the parent company of Triosyn Corporation) produces filters, for both individual and collective protection systems, containing the antimicrobial resin poly(styrene-4-[trimethylammonium]methyl triiodide) (PSTI). Taylor et al.[55] in 1970 showed PSTI to be an effective, broad-spectrum disinfectant in water-based solution. PSTI has been shown effective against threats including *E. coli*, *Giardia muris* and *G. lamblia*, Newcastle virus, polyomavirus, and a number of phages.[46,56–59] *In-vitro* results have suggested that air purification products incorporating PSTI provide a 99% increase in VRE compared to standard filtration systems.[31,60–62] PSTI air filters do appear to have some sensitivity to temperature and RH.[63] Ratnesar-Shumate et al.[64] have proposed that the mechanism of displacement of I_2 from the surface-bound I_3^- complex proposed in water by Taylor et al.[55] applies as well to bioaerosols undergoing near-contact with treated fibers during passage through an air filter medium. In this mechanism, microorganisms – which generally carry a negative charge on their outer membrane or coat – pass sufficiently close to the resin to displace an I_2 molecule from

the I_3^- complex. The I_2 molecule sticks to the microorganism and damages it, causing it to become non-viable. Since bioaerosols have higher surface charges than inert aerosols,[65] theoretically PSTI is a demand-release antimicrobial in air. However, the damage realized may depend on a number of external factors.

The measurement of the viability of microorganisms after passage through a PSTI filter is not entirely straightforward. Bioaerosols are often collected in impingers, but chemical species that off-gas from the filter also collect in the impinger fluid and may build up to toxic levels. Lee et al.[63] showed that PSTI can off-gas enough I_2 to cause the impinger liquid to become toxic to microorganisms. (PSTI is thus not exclusively a demand-release antimicrobial.) The test system cannot discriminate between killing of microbial agents in the aerosol state or in the impinger system. A proposed solution to this problem is the addition of reagents to the collection medium in the AGI (*e.g.,* sodium thiosulfate) to inactivate chemical species deposited in the collection fluid. Lee et al.[63] used this strategy when performing MS2 aerosol challenges of samples of PSTI media. They found that collecting in a thiosulfate solution effectively eliminated the increase in VRE over PRE, and collecting into a moderate excess (3%) of bovine serum albumin (BSA) caused only a 90% increase in VRE.

Eninger et al.[66] performed tests in which bioaerosols passing through a PSTI filter were collected into gelatin plates and measured no reduction in viable concentration caused by the PSTI. The absence of a reduction is likely a consequence of successful competition for aerosol-bound I_2 by the gelatin matrix. Rengasamy et al.[42] measured the survivability of MS2 virus captured within a PSTI filter to determine if it prevented the filter from becoming a fomite. They found that the PSTI filter did not cause a significantly

larger reduction in viability than a non-antimicrobial filter at low RH and temperature.[42] The lack of a difference may be because the dry capture surface of the antimicrobial filters lacks the activating agent needed by the deactivation mechanism, such as water.

Given this information, what would happen when a microorganism–iodine complex impacts the mucous membrane of a living being is not at all clear. The question remains: does a PSTI filter give an advantage in protection against infection by airborne pathogens compared to a non-treated filter? This question will be addressed by performing an animal exposure study in which challenges of a microbial agent will be delivered to age-, sex- and weight-matched test subjects in parallel experiments through a PSTI medium and through a mechanically matched inert medium. In this design the animal replaces the impinger as the detector. This planned experiment is expected to provide conclusive evidence that the incorporation of available I_2 at the surface of the air filter fiber does (or does not) convey clinical effectiveness to the medium.

Review of Animal Inhalation Exposure Systems

Reviews of experimental inhalation exposure systems have been performed by Drew and Laskin,[67] MacFarland,[68] Cheng and Moss,[69] Jaeger et al.,[70] Roy and Pitt,[18] Wong[71] and others. Inhalation exposures are generally performed when a researcher is studying an otherwise unquantifiable biological response – like measuring the MID_{50} of a microorganism – for which determination there is no useful *in-vitro* surrogate.

Usually an animal is used as a model of human respiration, the most common being the common laboratory rat (strains of *Rattus norvegicus*) and mouse (*Mus musculus*). Animal respiratory systems are imperfect approximations of the human respiratory system. For instance, the deposition efficiency in rat lungs reaches a minimum near 1 μm, while in the vastly different physiology of human lungs it is closer

to 100 nm.[71] Because of the risk of death and disease, high-risk experiments on humans are usually unethical if not outright illegal, and burdensome to perform when they can be morally justified. The Nuremberg Code of ethics for human experimentation, developed in response to atrocities committed in experiments on concentration-camp prisoners by German scientists within the Nazi regime, requires among other things that experiments on humans be based on the results of previous animal experiments.[72] Standard procedures for the ethical selection, care, and use of laboratory animals exist and are accepted by most (but by no means all) scientists.[73] These procedures mandate the use of the least-sentient animal that is appropriate for the experiment, thus the widespread use of laboratory rats and mice. Laboratory rodents are also, compared to other test animals, inexpensive to acquire and maintain.

Animal inhalation exposures of vapors appear in the scientific literature as far back as the late nineteenth century.[67] These early exposures were performed by putting the animal in a large container with room to move around and flowing the challenge into the animal's ambient atmosphere for it to breathe: this is called a whole-body exposure and is still in use. Whole-body exposures are a natural way to expose the animals and do not stress the animals by restraining them. Also, the animals can be housed in their exposure chamber, reducing contamination that may occur while transporting the animal. On the other hand, the animal can be exposed by other routes, such as dermal (the substance makes contact with the skin) or oral (the substances lands on or is absorbed into its fur and ingested when the animal grooms itself). Good air mixing in the chamber and a comparatively large amount of material is required, which may be problematic if the test material is highly hazardous.[71] Whole-body exposures have been

used on animals as small as rodents and as large as dogs,[67] and in the famous Operation Whitecoat, lasting from 1955 to 1973, the US Army performed experiments where humans were exposed to infectious bioaerosols in a 1-million-liter whole-body exposure chamber.[72]

Henderson, in 1952, made the important innovation, in the eponymous Henderson apparatus, of exposing only the nose and mouth of the animal to infectious aerosols, rather than the animal's whole body.[74] This kind of exposure is called a nose-only exposure or nose-and-mouth-only exposure. Nose-only exposures have the benefit of reducing the amount of material needed and eliminating the other routes of exposure in the animal. However, because the animals must be restrained, this method is not suitable for long exposures. The restraints stress the animals, and they may attempt to turn around in their restraints and accidentally suffocate themselves.[71]

A further distinction is made between nose-only exposures in which the animals breathe from a common plenum of air and exposures to the animals through individual air sources that are drawn out through a separate plenum. The first are called flow-past and the second are called directed-flow.[70] In flow-past systems the exhalations of animals earlier in the line are breathed by those further down: this may introduce undesirable variability.[71] An example of directed-flow exposures is the highly sophisticated system Baumgartner produced for the study of tobacco smoke in 1980.[75] Baumgartner is also responsible for designing the "Battelle" restraint tubes commonly used in nose-only exposure systems. Rihn et al.[76] validated a system for exposing mice to aerosolized asbestos fibers: he commented that studies on nose-only procedures for mice were, at the time he was writing in 1995, still fairly rare. Other nose-only aerosol

exposure systems have been produced for exposure to aerosols of radioisotopes,[77] asphalt fumes,[78] and pharmeceuticals,[79,80] as well as for general purposes.[70,81]

Experiments exposing animals to aerosols are common, as are experiments passing bioaerosols through filters; however, experiments using animal models to study the infectivity of filtered aerosols are not. Studies have been done on the reduction in infection that occurs when filters are added to the cages of pigs[82–84] and chickens[85,86] in an agricultural setting, but these studies examined casual transmission between animals and did not expose the animals to a metered challenge of aerosol. No reports of controlled exposures of an animal model of human respiration to an infectious bioaerosol penetrating a filter were found in the literature. Without an interaction like the PSTI–iodine chemistry problem described above, simple collection in impingers is sufficient and animal experiments are not necessary: that the PSTI animal study is the first to require such a system is not a surprise.

Exposure of a bioaerosol to the PSTI–iodine chemistry described above introduces the complications of time- and environment-dependent effects on the viability of microbial components of the aerosol. Because these effects can affect the claimed antimicrobial capability of the PSTI component of the fiber, measurement of the protective impact exerted by PSTI can be accomplished only with a biological indicator. Therefore, developing and characterizing a system to perform such an animal exposure experiment was necessary.

Factors Influencing an Animal Inhalation Exposure System

Before building the system, it was necessary to learn what to consider in the design of such a system. Wong[71] identified four key factors in which variability can adversely affect an animal inhalation study:

- individual response of the animal,
- animal environment,
- inhaled dose,
- exposure atmosphere.

The individual response of the animal is outside the scope of this work, as is a major component of the animal environment, the housing environment of the animals. The remainder of the animal environment is the exposure device used to expose the animals to an aerosol and their surroundings during the experiment. As described earlier, exposure devices can be whole-body or nose-and-mouth only. For the PSTI animal study, in which the MID_{50} can be reached after a relatively small period of time of exposure, a directed-flow nose-only system is appropriate.

The inhaled dose received by the animal can be calculated as Equation 1-2, wherein *C* is the concentration of test material in the animal's breathing air, V_m is the minute volume of the animal (breathing rate [in min^{-1}] times tidal volume), *F* is the fraction of material deposited, and *t* is the duration of exposure in minutes.[87] In a test of infectious bioaerosol, *C* is a concentration of airborne viable microorganisms (PFU/m^3 or CFU/m^3). The constants *C*, V_m, and *F* may vary in time. In reality, measuring these three quantities in real time is often not an option and instead these quantities are assumed to be constant. In the steady state, Equation 1-2 simplifies[71,87] to Equation 1-3. When making the steady-state assumption, as was done in this work, *F,* V_m, and *C*, and must be kept as constant in time as achievable.

$$D = \int_0^t C V_m F \mathrm{d}t \tag{1-2}$$

$$D = C V_m F t \tag{1-3}$$

The fractional deposition *F* depends on the PSD, and the sites in the animal's respiratory tract on which the particles deposit can affect infectivity. Even particles that

are not infectious can cause irritation or other effects that may affect the animal's respiratory system, resulting in swelling of membranes or increased mucus production: particles of one size may affect the deposition of particles of another size and the animal's immune response.[71] Variations in the PSD of the exposure atmosphere are a possible source of error in the dose.

The breathing rate V_m of an animal in laboratory conditions can vary wildly from its textbook values and be a source of error. Real-time measurements of respiration have been made on large animals, but no instances of real-time respiration measurements on mice can be found in the literature. Fairchild[88] reported that each mouse has a mean tidal volume of 0.18 mL and breathes 255 times a minute. However, in a laboratory situation, the breathing rate of an animal may vary widely from values recorded in less stressful situations, and flows from 1.5 to 10 times the total minute volume of the exposed animals have been recommended for nose-only systems.[71]

Obviously, variation in the airborne viable concentration C of infectious microorganisms is a source of variation in the dose. As mentioned in the introduction, the RH and temperature of a bioaerosol can affect its viability. Therefore, the system must keep the loss of viable particles due to humidity and temperature constant by keeping those factors consistent. In an animal exposure system, moderate loss of viability within the system can be tolerated as long as the viable concentration is consistent and the desired concentrations are attainable.

The aerosol source is another important potential source of variation in the PSD and viability. Wong[71] states that maintaining a stable concentration of aerosols is "notoriously more difficult" than other inhalation challenges. While that is certainly true

for fungi and dry powders, Collison nebulizers are often used to aerosolize a steady airborne concentration of viruses and bacteria from a liquid source in bioaerosol experiments: Hogan describes their use in animal tests as “almost exclusive.”[21] A steady Collison output depends on a steady feed pressure, as variations in pressure can alter the PSD.[16] In practice, evaporation of the nebulization liquid over time causes the aerosol concentration to increase with time at a slow rate. Also, because Collison nebulizers are recirculating systems that impose large shear forces, viability may decrease slowly.[17] Significant variation in output occurs among different models of Collisons.[89] This variation is not enough to change an experimental protocol, but using only one model of Collison in any series of experiments is wise.

Another factor that can affect the PSD of the challenge is the loading on the filter: as mentioned earlier, increased loading can increase Δp and can alter PRE. However, if the cumulative loading during the period of experimentation is low enough, changes in PRE and Δp are negligible. All these factors must be considered to keep an aerosol challenge consistent.

The gold standard for validation of an exposure system is to perform an exposure of animals and measure the consistency of the dose by measuring how much is deposited in the animal. This validation is more suitable for some exposures, where the dose is a static quantity that remains in the animal, than others. Directly performing this sort of validation is less helpful for challenges of infectious bioaerosols, because the microorganisms multiply when they reach a host.

Henderson measured the time-based coefficient of variation (CV) of viable concentration in the aerosol cloud in his apparatus and showed that within nine trials

using aerosolized *Bacillus subtilis* and 15 trials using *Chromobacterium prodigiosum*, the mean CV was 5.73% and no measured CV was above 15%.[74] Henderson remarked that this was very consistent and a number near Henderson's result was used as an objective in this work.

Objective

The hypothesis was proposed that an aerosol delivery system based on the Collison nebulizer can be designed and engineered to provide, at selectable concentrations, a respiratory challenge of bioaerosol particles that is verifiably consistent in time and that can be fed in separate experiments through treated and untreated control filters to deliver a consistent challenge to a small-animal model of human respiration. The goal of this work was to build an appropriate controlled animal exposure system, characterize the aerosol challenge delivered by the system, and validate the hypothesis that the challenge is sufficiently uniform to support statistically reliable animal infectivity testing. (Performing the animal exposure study was not a goal of this work; instead, it is enabled by this work.) The criteria for success initially set out were that within a number of individual experiments,

- from the time-dependent PSDs of the aerosol penetrating the filter, the TPCs, CMDs, and GSDs all have CVs less than 20%, and
- from time-series aerosol samples collected in the impingers, plated, and counted, the downstream airborne viable concentrations have a CV less than 20%.

CHAPTER 2
MATERIALS AND METHODOLOGY

Materials

Controlled Aerosol Test System

An aerosol delivery system based on the Collison nebulizer was designed and built. The system, called the Controlled Aerosol Test System (CATS) and illustrated in Figures 2-1 and 2-2, enables experiments measuring infection rates of a common laboratory mouse to discriminate the extent, if any, to which a treated air filter medium diminishes the exposure risk from an aerosolized pathogen challenge compared to the same challenge delivered through a mechanically equivalent untreated filter medium. The CATS generates a stream of biological aerosol at a range of constant concentrations, passes the aerosol through a filter, and delivers the penetrating particles to a mouse model of human respiration. Accommodation of the mouse model was a necessary aspect of the design and construction processes: it was also necessary that all components carrying aerosol flow fit within the biological safety cabinet where it will reside for the animal exposure trials. This cabinet is a SterilGARD III Advanced Animal Transfer Station (Baker Company, SG603-ATS), which has interior dimensions of 27 in H × 20 in D × 68 in W. The largest sash opening allowed when an infectious agent is present is 8 inches, which limits the reach of the operator(s). The convenience of the operator was considered in the design.

Tubing used to connect components containing aerosol flow is ½-inch stainless steel. All curves in the tubing containing the main aerosol flow are gradual and smooth, with an inner curvature radius greater than 1 inch. All valves carrying aerosol flow are ½-inch stainless ball valves. Flows of makeup and purge air are controlled by ¼-inch

needle valves, with toggle valves before the needle valves to enable the operator to turn flows off and on without having to readjust the needle valves. The needle valves are followed by rotameters to verify the flow rate. The Collison nebulizer is preceded by a toggle valve and rotameter. (The rotameter before the Collison is at pressure and will read lower than its actual flow: it can be corrected using Equation 2.51 in Hinds.[90] At a Collison pressure of 25 psig the rotameter can be corrected by multiplying its reading by 1.64: at 30 psig, 1.74.)

In the CATS, air is supplied to the system by an air compressor. For this work the lab air line was filtered first through an oil trap and then a DFC-21 HEPA canister particle trap (Porous Media Corp., St. Paul, MN) to feed the nebulizer and porous tube diluters. The animal studies require a source of breathable air free of both particles and toxic gases and vapors, to be provided onsite.

The incoming air is then regulated to a pressure of 50 psig and flowed through a porous tube humidifier (PermaPure LLC, Toms River, NJ; model MH-070), which contains a Nafion® membrane tube. Water on the outside of the tube is transported through the membrane into the air flow. The humidity can be controlled via the water temperature, although water at a different temperature than ambient was not needed in this work. If a low RH is needed the tube can be removed, although evaporation from the Collison nebulizer increases the humidity of the aerosol flow somewhat.

The airflow is then manifolded. Some of the airflow is regulated to 30 psig and flowed into the system later on. The rest of the flow is regulated to 25 to 30 psig and flowed to a Collison nebulizer (BGI Inc., Waltham, MA), which generates the bioaerosol. A single-jet Collison nebulizer is used because it does not produce excessive flow.

Make-up air supplies the rest of the flow. A 1-psig pop valve to prevent overpressure and a pressure gauge (Dwyer Instruments, Houston, TX; Magnehelic series 2000) tee off directly after the Collison. A porous tube diluter (Mott Corp., Farmington, CT; model #7610105-020) is used to deliver make-up air from Valve A to adjust the flow rate after the nebulizer. The porous tube lets the two air flows mix in a non-turbulent fashion.

The nebulization process puts charges on the created particles. A charge neutralizer (TSI Inc., Shoreview, MN; Model #3012A) is necessary to neutralize that electrical charge. The 3012A charge neutralizer uses a 370-MBq ^{85}Kr beta-emitting source. It can be used with flows as high as 50 L/min. After the charge neutralizer, a length of tubing guides the flow to an intersection where the first sampling point in the system, Valve and Port 1, tees off and can be connected via ½-inch conductive silicone tubing (TSI, part #3001789) to sampling instrumentation. A type of O-ring compression fittings known as “Ultra-Torr” hose connectors are present at the sampling ports to allow the operator to easily connect and disconnect instrumentation.

After this tee is a custom-built sample holder (Triosyn Corp, Williston, VT) comprising an inner and outer sleeve holding a 47-mm diameter disc (40-mm exposed) of filter medium compressed (by bolts around the edges) between elastomeric annular seals. The sleeve has a tapered chamber 10 cm long before the filter to allow the aerosol to spread, and then a tapered chamber 10 cm long after the filter to return to the tubing. The holder can accommodate other sizes of filter with the use of reducers, although reducers were not used in these experiments.

A second sampling point is directly downstream of the sample holder at Valve and Port 2. A differential pressure gauge (Dwyer, Magnehelic series 2000) is connected

before and after the sample holder to measure Δ*p* across the filter. Valve 3, following the tee for Valve 2, is necessary to isolate the aerosol flow from the animal subjects during post-exposure samplings of the aerosol. Downstream of Valve 3, flow from Valve B can be supplied immediately after the exposure is terminated to give the animals clean breathing air before their removal from the exposure system.

Next, the exposure system, a Jaeger–NYU Modular Nose-Only Directed-Flow Rodent Inhalation Exposure Unit (CH Technologies, Westwood, NJ),[91] hereinafter referred to as a mouse tree, is used to expose the mice to the aerosolized agent. A nose-only system was, among other reasons, chosen to prevent cutaneous and enteric infections to the mice. The capacity of the mouse tree to deliver infectious aerosol to mice was not tested in this work. The mouse tree itself has been validated and verified in the literature by Jaeger, so repeating that process is not necessary.[70]

Each mouse is placed in a polycarbonate holder and constrained with a sealed restraint inserted in the rear opening of the holder so that the tip of the mouse's nose projects out of an opening in the front of the holder. The holder inserts securely into a socket on the mouse tree. Vents inside the body of the tree blow an airstream containing the filtered aerosol at the nares of the mouse as its only source of breathing air. Exhaled air and excess flow are drawn away from the mouse.[70] The mouse tree is a directed-flow system and no mouse rebreathes flow from other mice. The mouse tree can hold up to 12 mice at one time. A rotating joint is present at the inlet to the mouse tree to allow it full range of rotation and make all the sockets accessible.

At the effluent of the mouse tree, the relative humidity and temperature are measured by a National Institute of Standards and Technology (NIST)-traceable digital

hygrometer (Control Company, Friendswood, TX; Model 35519-020). The flow may either be sampled at Valve and Port 4 or exhausted through another HEPA canister filter, after which a flow meter (TSI, Model #4143D) measures the flow rate.

Sampling Instrumentation

All-glass impingers

Built into the CATS is a hook-up for sampling with impingers. Sampled aerosol flow is combined with flow from needle Valve C in another porous tube diluter. This combined flow is drawn through Valve and Port 5a or 5b into AGI-4 impingers (Ace Glass Inc., Vineland, NJ). When a vacuum is drawn on AGI-4s, they draw 12.5 L/min of air.[21] Therefore the rate of sampling from the system is controlled by the make-up air from Valve C, which is usually set to 10 to 11.5 L/min (i.e., 1 to 2.5 L/min of aerosol sampling). The efficiency of an impinger decreases with time because of evaporation and aerosolization of the sampling liquid.[21] Therefore, two impingers must be present in the system so that the operator can switch to a fresh impinger after a time and replace the used impinger base with a fresh one. Only one impinger is in use at any given time, although when switching between the two, Valves 5a and 5b may be open simultaneously. A vacuum pump is used to draw air through the impingers, and thence through a HEPA filter to capture uncollected aerosol. The pump is a generic component and, being downstream of the HEPA trap, it is not a potential source of contamination.

Particle sizers

The operator can also make measurements using particle sizers. Two different instruments are used to measure PSDs. For particles in the micrometer range, such as most bacteria, an aerodynamic particle sizer (APS) is used. For particles in the nanometer range, such as viruses, a scanning mobility particle sizer (SMPS) is used.

The APS (TSI, Inc.; Model 3321) operates by measuring the time of flight of particles accelerated through a nozzle. The acceleration is measured by parallel lasers. Particles from 0.5 µm to 20 µm can be sized by the APS. The APS samples at a flow rate of 5 L/min and can sample continuously.

TSI's SMPS consists of a Model 3080 electrostatic classifier with a 3081 long differential mobility analyzer and a 3785 condensation particle counter. The 3080 and 3081 separate particles based on their electrical mobility. The separated particles pass into the 3785, which "grows" the particles by condensation of water vapor and counts the resulting droplets optically. Particles from 10 nm to 1 µm can be sized with the SMPS. The length of the particle sizer's sampling interval depends on the total concentration of particles in the air; lower concentrations require more sampling time to get sufficient particle counts. The particle range depends on the sampling rate. At 0.6 L/min, particles with diameters from 10 to 410 nm can be measured, and other size ranges require a sampling rate of similar magnitude.

Challenge Microorganisms

Using a challenge of bioaerosol was necessary to validate the system, but as the system was not contained in a biological safety cabinet for this work, infectious bioaerosols were not an option. Two nonpathogenic microorganisms were chosen for this work: MS2 coli phage virus and *Bacillus atrophaeus* bacterial spores.

MS2 coli phage

MS2 bacteriophage is a small RNA virus that lives on male cells of *E. coli* bacteria. It has an icosahedral virion with a diameter[92] of about 27 nm. Its coat does not have a lipid layer, thus in theory making it more stable at high RH.[11,92] Trouwborst et al.[93] showed that if MS2 is nebulized from a fluid with appropriate concentrations of protein

and salts, over an interval of 30 minutes it loses less than one order of magnitude of viability, regardless of RH. Again, that the challenge stay consistent is more important than that it not lose any viability, and since this MS2 stock grew at a titer of 10^{11} to 10^{13} PFU/mL, one order of magnitude of loss poses no problem. MS2 is a common simulant for infectious viruses. Because keeping MS2 viable in the aerosol state is comparatively easy, it has been used in a large number of studies.[21,25,31,32,37,39,42,50,61,63,66,93]

Stock of MS2 virus, from American Type Culture Collection (ATCC) 15597-B1, was grown in *E. coli* (ATCC 15597) in tryptic soy broth (TSB) according to standard EPA protocols.[94] To determine viability, a single-layer plaque assay was performed.[94] In this assay, a 1-mL portion of a serial dilution of the impinger aliquot was combined with 250 µL of an *E. coli* stock and 9 mL of warm, liquefied tryptic soy agar (TSA). This mixture was poured into an empty Petri dish and left to cool and solidify, and then the plates were incubated overnight. Plaques were counted the next day.

Bacillus atrophaeus

The genus *Bacillus* makes up a variety of endospore-forming, Gram-positive rod-shaped bacteria. *Bacillus* spores are resistant to air-drying and other stresses, and therefore can be found in a wide variety of environments.[95] *B. anthracis* is the causative agent of the disease anthrax and is of concern in bioterrorism defense. Some non-infective *Bacillus* species are often used as simulants in bioaerosol tests, because of their hardiness and their similarity to *B. anthracis*.[22–24,61,62,65,74,96]

B. atrophaeus is virtually identical to the common species *B. subtilis*, or “hay *Bacillus*,” except that *B. atrophaeus* produces a black pigment on media containing an

organic nitrogen source.[97] *Bacillus* cells range from 0.5 µm D × 1.2 µm L to 2.5 µm × 10 µm: *B. subtilis* and *B. atrophaeus* are on the small end of this range.[95]

B. atrophaeus spores, from ATCC 9372 stock, were grown in TSB according to standard methods,[98] at a titer of approximately 10^8 CFU/mL. Samples containing *B. atrophaeus* were applied with a spiral plater (Microbiology International, Frederick, MD) onto TSA plates and then incubated overnight. Colonies were counted the next day.

Filter Media

Three different filter media, extracted from FFRs available on the market, were used in these consistency tests. Samples were taken from these filters using a 47-mm circular punch and mallet. An off-the-shelf HVAC filter rated at Minimum Efficiency Reporting Value (MERV) 8 was also used, but it was found to have PRE near zero in the size range considered in this work, so it was discarded.

Safe Life T-5000

The Safe Life T-5000 FFR is a NIOSH-certified P95 respirator. This filter was chosen because it contains the PSTI resin and is therefore similar to the filters that will be used in the animal exposure study. A flow rate through the filter of 5.3 L/min was used because Safe Life Corp. specified for the animal experiment a testing face velocity for their material of 7.08 cm/s. At the 85-L/min flow rate used by NIOSH for testing FFRs,[35] this face velocity scales to an FFR with a 200-cm^2 surface area, which is a reasonable estimate of the true area. (A rough measurement with a ruler of a T-5000 mask gives 160 cm^2.)

Measurements of the T-5000, either the PRE of the fabric or the protection of the FFR when worn, do not seem to exist in the literature. Ratnesar-Shumate et al.[64] measured the PRE and VRE of Safe Life-produced P95 filter fabric, although their fabric

was designed for respirator cartridges, not FFRs. In their work, fluorescent particles with a mass mean diameter of 0.27 µm were removed with PRE near 99%. The bacteria *E. coli* and *Micrococcus luteus* were removed with almost five nines of VRE.[64]

The T-5000 consists of a covering outer layer, a layer of electrically charged polypropylene filtration material embedded with particles of PSTI, a carbon layer to reduce organic vapors, and a supporting inner layer. Only the filtration layer was used.

3M 1860S

The 3M Corporation produces N95 particulate respirators that are commercially available in most hardware stores. Model 1860S was used in this work, as an alternative to the T-5000. Coffey reports this respirator as, when worn with a correct fit, filtering with 95% PRE among 90% of wearers.[38] However, measurements of the efficiency of the filter fabric itself do not seem to exist in the literature. The filter media of the 1860S is an electret made of polypropylene fibers.

Isopropyl alcohol-treated 1860S

Exposing electrically charged filter material to vapors of isopropyl alcohol (IPA) removes the electrical charge and decreases the filter's PRE. One swatch from an 1860S was exposed to vapors of IPA to enhance penetration.

Methods

Leak Check

After the system's construction was finished, it was leak tested by replacing the Collison nebulizer with a plug and pressurizing the system to a few inches of water, then observing it for an hour. If no significant change occurred, the system was deemed leak-free. A full description of this leak check procedure is in Appendix A.

Flow Rate, Relative Humidity, and Temperature Consistency

To determine the consistency of flow rate, RH, and temperature, the system was operated with deionized (DI) water as the Collison liquid. The flow rate at the exhaust and the RH and temperature were recorded over a period longer than an hour. No filter was used in this test.

Correlation of Sampling Ports

To determine the loss of particles during flow in the system, and to make sure samples from different ports could be compared with one another, a correlation of sampling ports on the instrument was performed by nebulizing two separate suspensions, one of 250-nm polystyrene latex (PSL) beads (Duke Scientific, Palo Alto, CA; G250) and one of 1-µm PSL beads (Duke Scientific, 4009A). Beads were dispersed in DI water (on the order of one unit of bead solution to ten units of water) as the Collison nebulization liquid. The makeup flow was adjusted to deliver a total flow of 5.3 L/min, and the system was allowed to equilibrate. No filter was used in this test.

Each of the sampling ports (1, 2, and 4) and the ports on the impinger hook-up (5a and 5b) were sampled repeatedly with the particle sizer, as were ports on each quadrant of the mouse tree. For readings from the mouse tree, the sampling tube was inserted into a mouse restraint device and inserted into the sockets of the tree at four different quadrants. For Ports 5a and 5b on the impinger hook-up no dilution air was added: Valve C was closed. The dilution air would be too much for the particle sizer to sample and some would need to be vented by opening the alternate port to prevent backflow. Whether venting would entirely prevent backflow was unclear, and when the test was attempted with venting, the readings deviated wildly from what was expected.

For the 250-nm beads, readings were taken in triplicate at each port and the mean of those three readings was used. For 1-µm beads, only one reading was taken at a time. The consistency was calculated based on the combined concentration at the aerodynamic diameter where the peak occurred and the two surrounding data points.

Bioaerosol Consistency Trials

To test the consistency of the challenge delivered, a bioaerosol was created and flowed through the system. Microorganism stocks were individually diluted in filter-sterilized water and delivered into the Collison nebulizer. The Collison spray was started and the make-up flow was adjusted to deliver a total flow of 5.3 L/min. The system was allowed to equilibrate for 15 minutes before 5-minute impinger samples into 1X phosphate-buffered saline (PBS) were taken at sampling ports 1 and 2, and particle size measurements were taken at port 1. Particle counts at port 2 were too close to the instrument error to be useful for measuring consistency, though downstream measurements were made to verify filter integrity. Δp was observed throughout the experiment. A step-by-step operating sequence for these experiments are in Appendix A: a specific sequence of movements was used to prevent splash from the impingers.

The first series of tests nebulized 30 mL suspensions of MS2 virus with nominal titers ranging from 10^8 to 10^{12} PFU/mL. (The nominal titer is the concentration of viable microorganisms in the liquid, calculated based on the original titer of the stock and the dilution ratio.) For each test in this series, T-5000 medium was used. Pairs of particle sizer measurements upstream of the filter concurrent with an impinger collection downstream alternated with downstream sampling into impingers: thus, two upstream PSD measurements and a downstream impinger sample were made during the first, third and fifth 5-minute sampling period, and upstream collections into impingers were

made during the second, fourth and sixth periods, resulting in a total of six values for PSD and three each for viable counts before and after the filter. The SMPS scanning period was consistently 135 seconds long. Impingers sampled 1.5 L/min of aerosol flow with makeup air to increase the flow rate to 12.5 L/min. Total sampling time was a bit more than 30 minutes because switching between impingers was labor intensive.

A second series of tests nebulized 16 to 30 mL suspensions of *B. atrophaeus* spores with nominal titers of 10^7 to 8×10^7 CFU/mL. Media used were T-5000, 1860S, and IPA-exposed 1860S. The impingers sampled at a rate of 2.5 L/min of aerosol flow during the first three experiments and at 1.5 L/min during the final three, with makeup air to increase the flow rate to a total 12.5 L/min. Because the APS draws more air than the SMPS, it could not be operated an the same time as an impinger; therefore, particle size measurements were taken before and after impinger measurements, resulting in seven particle size measurements (with sometimes an extra initial measurement) and three viable counts at each sampling point per experiment. The APS sampling period was 20 seconds. The total length of an experiment was about 40 minutes.

After the experiments, the impinger media were serially diluted and plated in triplicate, and incubated overnight at 37 °C. Plaques or colonies were counted the next day. The remaining liquid in the Collison nebulizer was also plated in triplicate and counted. The dilution series deemed to be the smallest dilution that was not too overgrown to be reliable (generally, fewer than 60 CFU/plate or PFU/plate) was used to calculate the concentration. The titer of the Collison liquid was calculated by Equation 2-1, where N is the count of CFU or PFU on the plate, V_p is the volume of liquid plated (always 1 mL), and n is the dilution factor. The airborne viable

concentration was calculated from the plates by Equation 2-2, where V_i is the volume of impinger liquid (in this work always 20 mL), Q_a is the flow rate of aerosol flow collected (1.5 to 2.5 L/min) and t is the duration of sampling (in this work always 5 minutes).

$$C = \frac{N}{10^{-n}\, V_p} \tag{2-1}$$

$$C = \frac{N\, V_i}{10^{-n}\, V_p\, Q_a\, t} \tag{2-2}$$

Penetration curves were calculated for select experiments; for each individual aerodynamic diameter, the mean of upstream readings and the mean of downstream readings were plugged into Equation 1-1. Confidence intervals were calculated based on the combined standard deviation, assuming a normal distribution. The combined standard deviation is calculated as per Equation 3.18 in Taylor[99] for uncertainties of ratios, using the standard deviation of upstream and downstream readings.

From each time step, the mean and standard deviation of the TPC, CMD, and GSD of the aerosol distribution and the airborne viable concentration was calculated. For each individual experiment, the CV is calculated as the ratio of standard deviation of the time-based data points to the mean. CVs are calculated for each individual experiment: data is not pooled between experiments. The aerosol need only remain consistent within an experiment, not between experiments. For the experiments with the largest and smallest GSD, the CV of the regular standard deviation is also calculated, to demonstrate that the standard deviation does not grow wildly compared to the GSD.

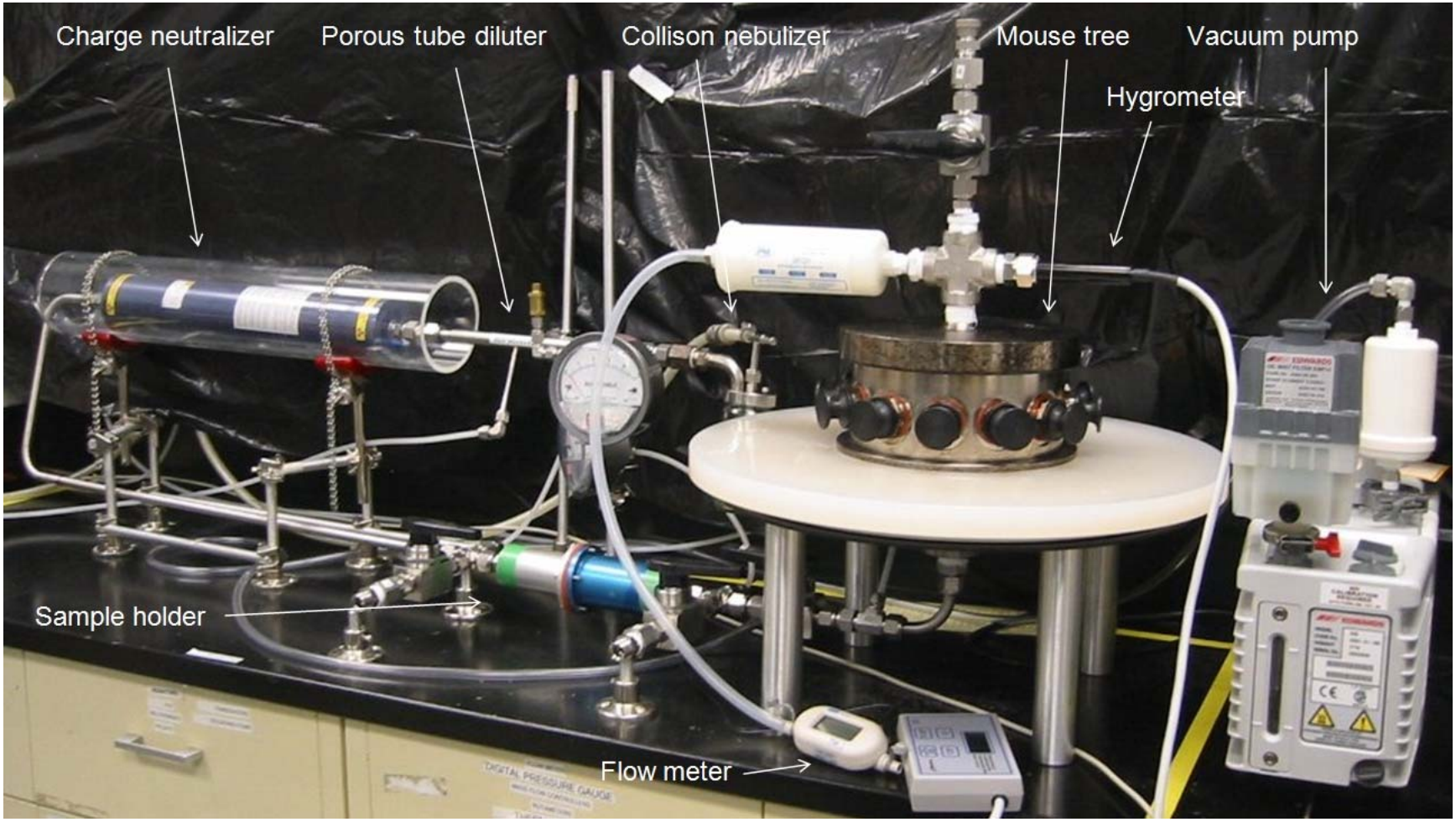

Figure 2-1. Photograph of Controlled Aerosol Test System (CATS), with key components labeled. Not pictured: control panel and impinger hook-up.

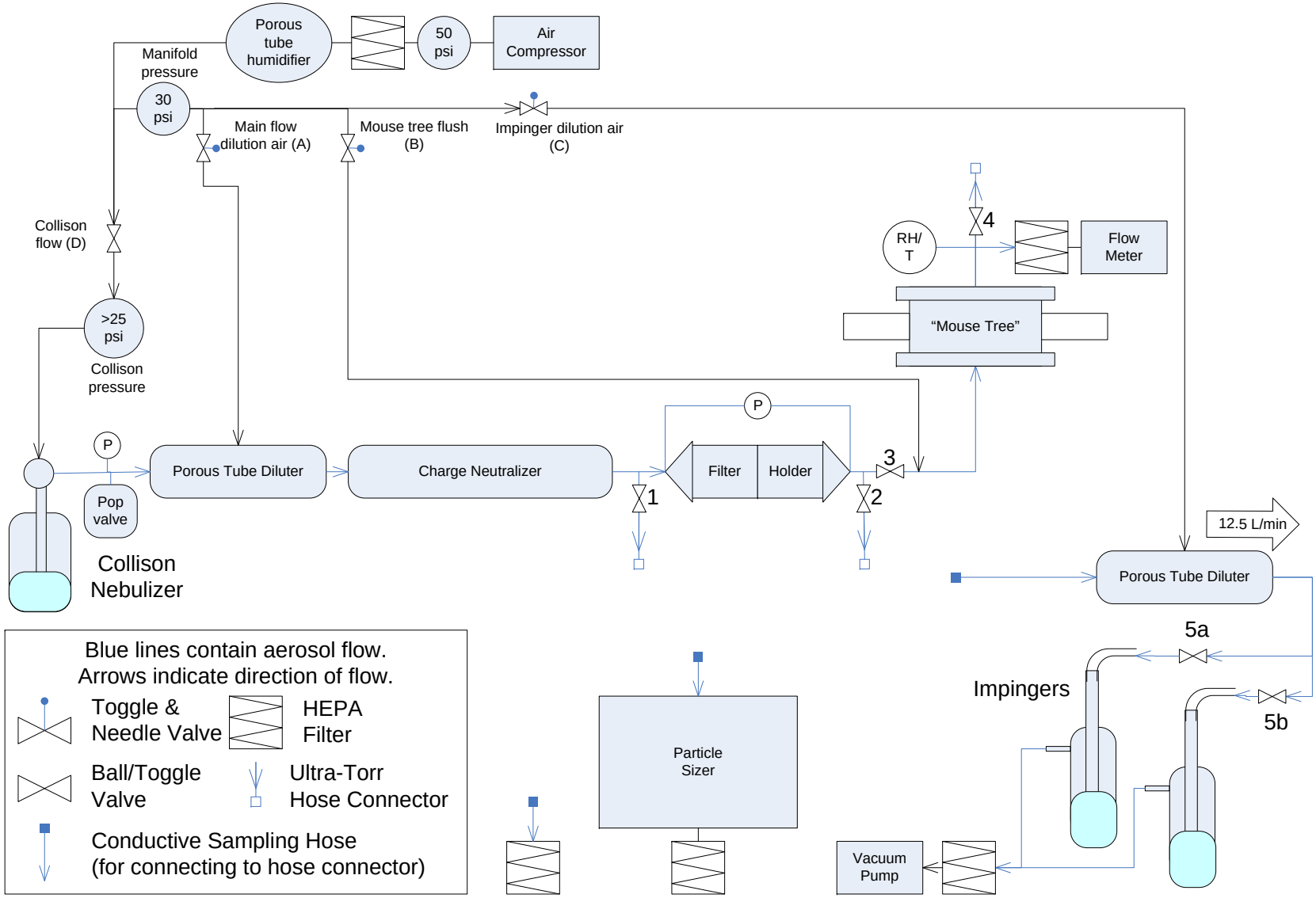

Figure 2-2. Process-flow diagram of CATS.

CHAPTER 3
RESULTS

Leak Check and Flow Rate, Relative Humidity, and Temperature Consistency

The system was leak checked: it held 3 in H_2O of pressure for an hour. Then the consistency of the flow rate, humidity, and temperature was measured. Deviations from the mean for the temperature and the exhaust flow rate were lower than 1% for observations during a 90-minute period, and RH stayed within 5%. Toggling Valve C to turn flow to the impingers on or off caused a deviation in flow of about 2%. Data for this are presented in Appendix B.

Correlation of Sampling Ports

Data for the port consistency trials performed with inert beads are presented in Appendix C. For 250-nm beads, the worst case difference between ports is 15% of the overall mean, but the minimum value was taken at the beginning of the system (Port 1) and the maximum at the end (Port 4), which is the opposite of what would be expected if particles were being lost along the length of the system, and readings that large did not occur more than once. All deviations remained within 10% of the overall mean. For 1-µm beads, the worst-case difference between ports was 4.8% of the overall mean, and the worst deviation from the mean was 2.7%, which is very consistent.

Bioaerosol Consistency Trials with MS2

The PSD was observed to be approximately log-normal: a representative plot is given in Figure 3-1. Although the PSD measurements on the downstream side of the filter were not usable for consistency, they could be compared to the upstream measurements to calculate a penetration curve, as in Figure 3-2. With 95% confidence, it can be said that the PRE of the T-5000 in the 10 to 400 nm range is between 99.77%

and 99.97%, and the MPPS is likely somewhere between 100 and 300 nm. This PRE is higher than that measured by Ratnesar-Shumate et al.,[64] who were using P95 material from cartridge respirators, not FFRs: whether the same material is used in Safe Life's FFRs and their cartridges is unknown. However, given the high titer of the microorganism, viable penetration was assumed to be possible. Plated viable MS2 counts were not measurable, likely because of problems executing the assay method or contamination in the laboratory workspace.

Mean values of RH, *T*, and the TPC, CMD, and GSD of the PSD are presented in Table 3-1, as well as the coefficients of variation (CVs) of the PSD moments within each experiment. Raw data for this series of experiments are in Appendix D. The PSD varied very little over the 30 minutes observed, as reflected in the very low CVs of the moments, all 6% or less. The PSD moments were not observed to trend upwards or downwards in time during the 30 minutes of observation. No noticeable change in Δp was observed over the course of the experiments.

Bioaerosol Consistency Trials with *B. atrophaeus*

The PSD produced by aerosolizing B. atrophaeus was observed to be bimodal, with particles in the peak near 1 µm containing bacteria, and a hump of smaller particles presumed to contain only dissolved solids from the aerosolization medium. Based on the representative distribution in Figure 3-3, the dividing point between the two modes was taken as 0.8 µm, the concentration of particles larger than 0.8 µm was calculated, and the CV of that concentration was measured as well.

For the T-5000 medium, penetration in the range measured by the APS was so small as to be indistinguishable from instrument noise. Because of this, the 1860S medium was tried: the downstream data were still unusably small. The IPA-treated

1860S medium was less efficient: the PSD measured downstream of this filter is shown in Figure 3-4, and its curve of PRE versus particle size is reproduced in Figure 3-5. With 95% confidence, it can be said that its PRE near 1 µm was still no lower than 99.95%. Regardless of this, these experiments were attempted anyways, in the hopes that enough bioaerosol would penetrate over the sampling period to be measurable. Viable counts of microorganisms were measured upstream, but no viable microorganisms were detected downstream – even past the IPA-treated 1860S – because the challenge concentrations were not large enough to overcome the high filtration efficiency of these filters at particle sizes near and above 1 µm. Plates from downstream samples showed an occasional lone colony, not enough to calculate from reliably.

Table 3-2 identifies the filter used in each test, lists the mean values of RH and temperature, and reports values of the TPC, CMD, and GSD of the PSD. Table 3-3 contains the nominal titer in the Collison pre-experiment, the post-experiment Collison titer, the mean upstream viable concentration, and the CVs of the PSD moments and upstream airborne viable concentration measured within the experiment. No trend upwards or downwards was observed in the PSD moments. Raw data for this series of experiments are reported in Appendix D.

No change in Δp was observed over the course of any experiment in this series. Downstream measurements were performed before and after the exposure of the IPA-treated 1860S medium, as shown in Figure 3-4. While there appears to be more penetration after the exposure, the increase is within the error of the APS.

By performing a linear regression between the post-experiment titer of the Collison liquid and the airborne viable concentration, the VSF for the CATS was determined to

be 7.8×10^{-7}. This linear regression had a R^2 of 90%. Note that this VSF does not account for the viable collection efficiency of the impingers: if it did, it would be somewhat larger. Also note that this spray factor is for the entire system: the VSF at the Collison nozzle can be calculated to be about 2×10^{-6} by scaling by the ratio of total flow to Collison flow (5.3/2). The TPC correlated well with the TPC larger than 0.8 µm, with an R^2 higher than 99% and a regression constant of 0.4996. In Experiment 901 and the ones following, the post-exposure Collison titer seems to be depressed by an order of magnitude compared to the nominal titer, while in experiments before 901 the concentrations are of the same magnitude. Neither the TPC nor TPC above 0.8 µm showed an obvious correlation with the post-experiment Collison titer or the airborne viable concentration, although both TPC and TPC above 0.8 µm correlate with the nominal Collison titer with R^2 near 99% (with regression constants of 10^{-5} and 7×10^{-6} #/CFU, respectively).

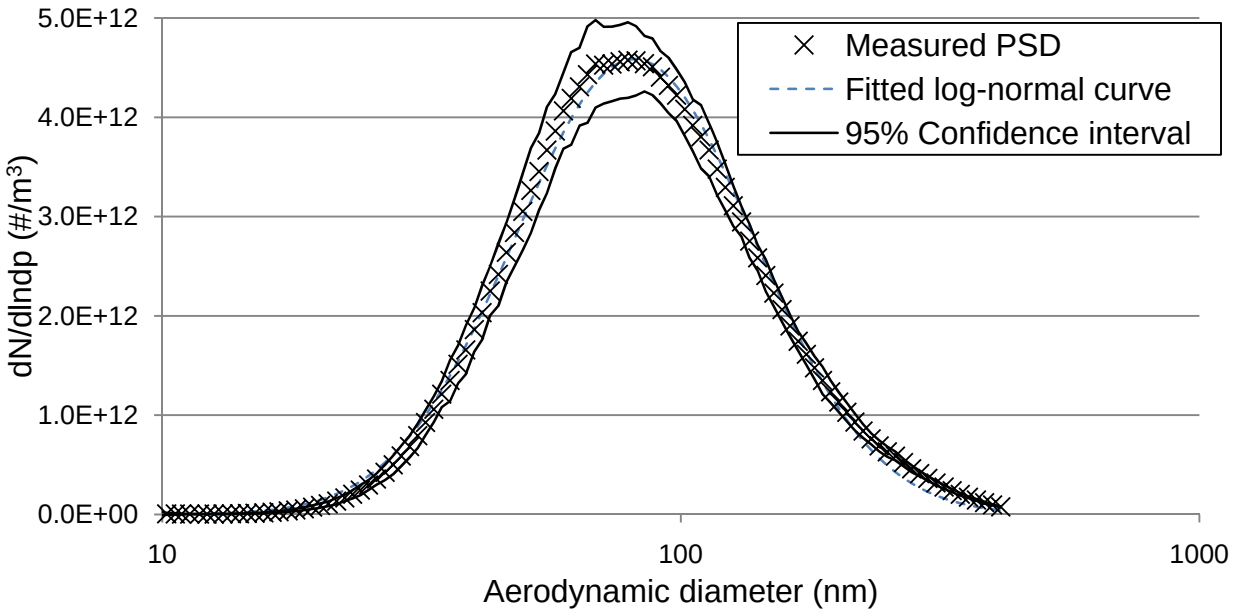

Figure 3-1. Representative particle size distribution (PSD) from MS2 nebulization and 95% confidence intervals for each individual diameter. Based on six samples in Experiment 724.

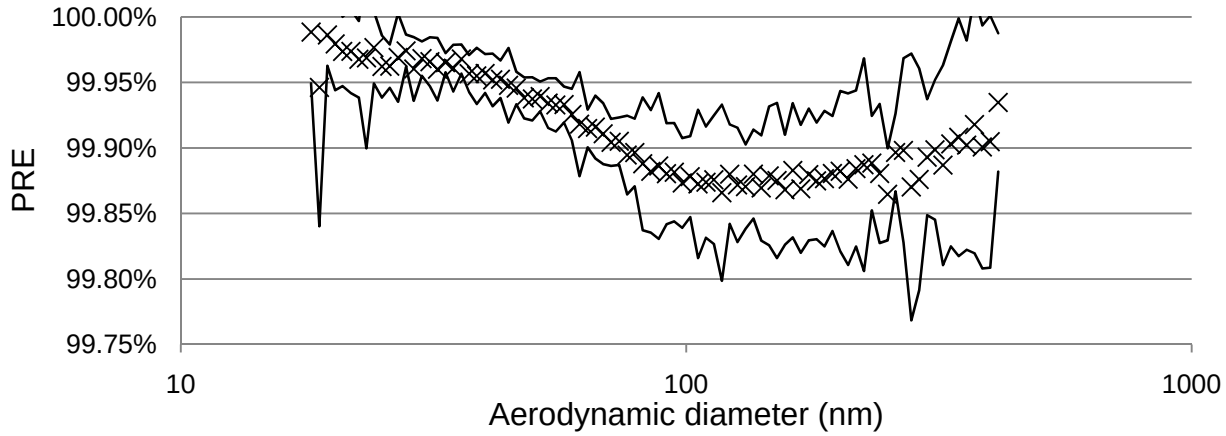

Figure 3-2. Particle removal efficiency (PRE) of T-5000 medium as a function of particle size and 95% confidence intervals. Calculated from six upstream samples and three downstream samples in Experiment 811.

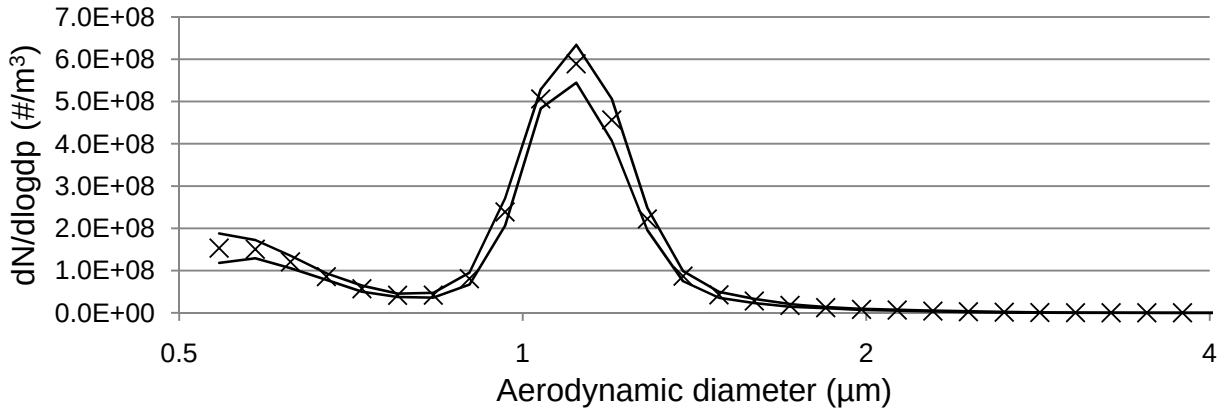

Figure 3-3. Representative PSD from *Bacillus atrophaeus* nebulization and 95% confidence intervals. Taken from seven samples in Experiment 819.

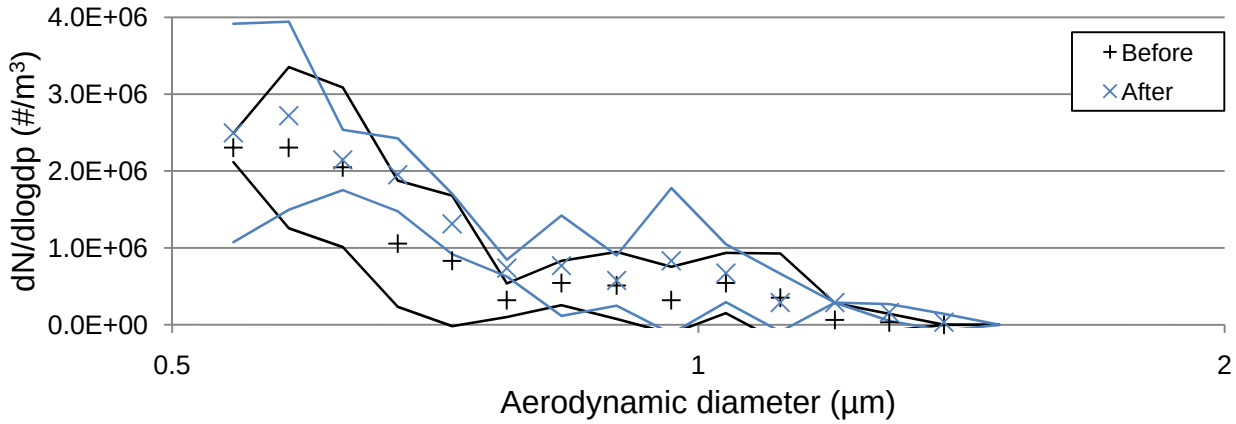

Figure 3-4. Downstream measurements from Experiment 910 with isopropyl alcohol (IPA)-treated 1860S medium and 95% confidence intervals. Three samples each were taken before and after the 40 minutes of experimental interval.

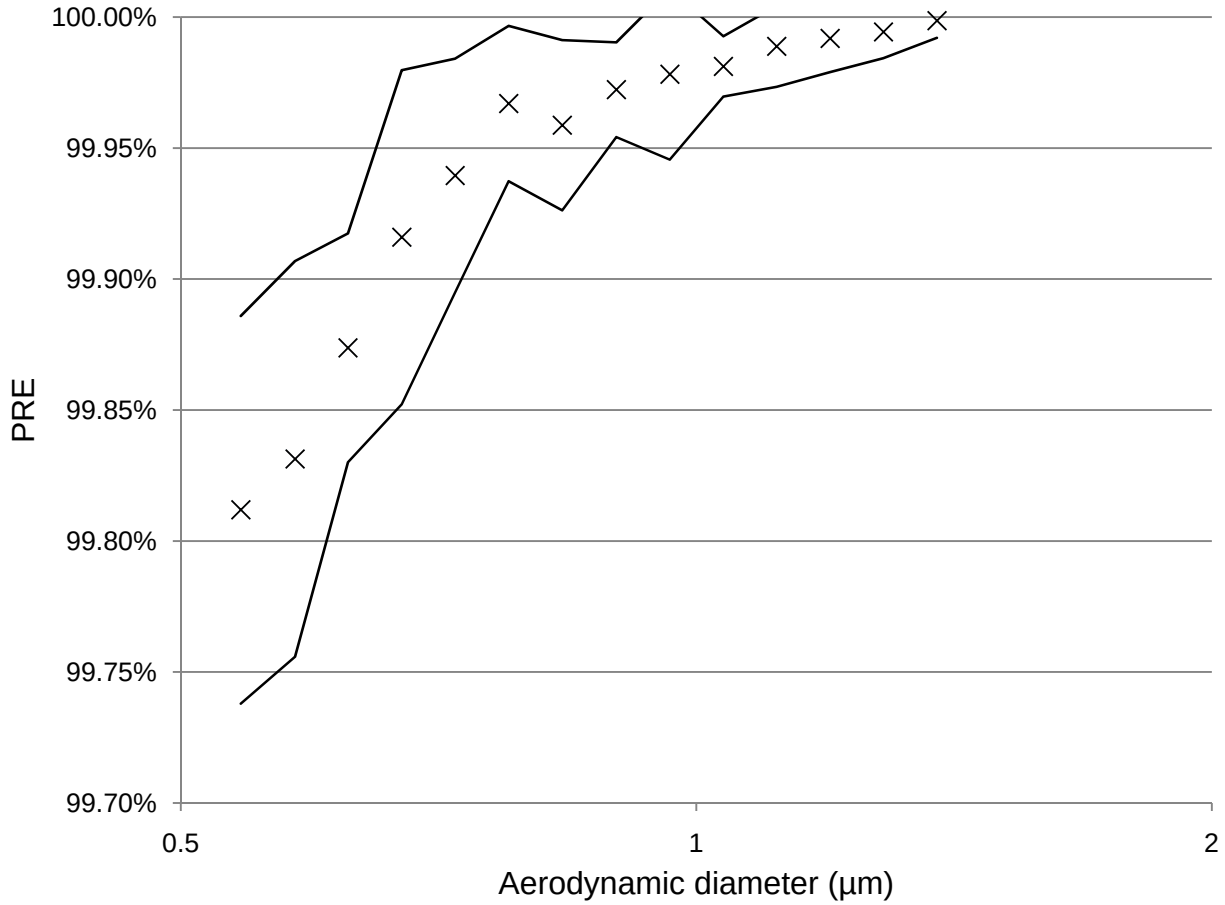

Figure 3-5. PRE of IPA-treated 1860S medium as a function of particle size and 95% confidence intervals. Calculated from seven upstream samples and six downstream samples in Experiment 910.

Table 3-1. Mean relative humidity (RH), temperature, and particle size distribution (PSD) moments, and coefficients of variation (CVs) of PSD moments for MS2 experiments

			TPC			CV of		
Experiment	RH	T (°C)	(10^{12} #/m^3)	CMD (nm)	GSD	TPC	CMD	GSD
724	65%	ND[a]	2.65	81.41	1.70	4.66%	1.23%	0.19%
728	61%	22.7	4.51	74.22	1.69	5.21%	3.09%	1.02%
730	64%	22.4	4.16	75.72	1.68	3.31%	1.20%	0.21%
811	55%	27.0	5.13	75.62	1.72	3.16%	1.93%	0.56%
812	53%	26.5	4.51	76.53	1.70	6.00%	0.49%	0.08%
813	56%	26.0	4.11	77.96	1.70	5.15%	0.37%	0.29%
Minimum	53%	22.4	2.65	74.22	1.68	3.16%	0.37%	0.08%
Maximum	65%	27.0	5.13	81.41	1.72	6.00%	3.09%	1.02%
Mean	59%	24.9	4.18	76.91	1.70	4.58%	1.38%	0.39%

[a] No data. The maximums are simply the largest entry of data in the above column. Minimums are, similarly, the smallest entry. The means are simply the mean of the data in the corresponding column. CVs are calculated within each experiment. The CVs of standard deviation for Experiments 728 and 812 are 0.59% and 0.47%, respectively.

Table 3-2. Filter used, mean temperature, RH, and PSD moments for *Bacillus atrophaeus* experiments

Exp.	Filter	RH	T (°C)	TPC (10^6 #/m^3)	TPC > 0.8 µm (10^6 #/m^3)	CMD (µm)	GSD
819	T-5000	58%	24	123	73.3	1.08	1.33
820	T-5000	57%	24	59.2	49.3	1.10	1.21
827	1860S	63%	23	30.8	6.56	0.705	1.35
901	1860S	50%	23	254	121	0.953	1.33
903	1860S	48%	23	259	120	0.942	1.34
908	1860S	47%	22	528	254	0.948	1.34
909	1860S	47%	23	1208	580	0.951	1.34
910	IPA 1860S	45%	23	1174	590	0.960	1.34
Minimum		45%	22	30.8	6.56	0.705	1.21
Maximum		63%	24	1208	590	1.10	1.35
Mean		52%	23	455	224	0.955	1.32

The lower portion of the table is calculated the same as Table 3-1.

Table 3-3. Viable concentrations and CVs of PSD moments and upstream airborne viable concentration for *B. atrophaeus* experiments

	Collison (10^6 CFU/mL)		Upstream	CV of				
Exp.	Nominal	Post-exp.	(10^6 CFU/m^3)	TPC	TPC > 0.8 µm	CMD	GSD	Upstream
819	10	26.3	23.3	5.21%	3.12%	0.69%	0.42%	19.21%
820	10	14.0	7.15	7.42%	5.26%	0.60%	0.58%	24.02%
827	10	ND[a]	ND[a]	9.77%	9.66%	6.18%	0.77%	ND[a]
901	16	2.80	3.04	6.81%	3.64%	0.70%	0.15%	18.23%
903	16	ND[a]	ND[a]	5.00%	4.58%	0.35%	0.08%	ND[a]
908	40	5.57	5.10	3.84%	4.21%	0.42%	0.22%	25.54%
909	80	ND[a]	ND[a]	9.21%	4.97%	0.91%	0.19%	ND[a]
910	80	18.0	12.4	3.52%	4.94%	0.30%	0.14%	5.00%
Min.	10	2.80	3.04	3.52%	3.12%	0.30%	0.08%	5.00%
Max.	80	26.3	23.3	9.77%	9.66%	6.18%	0.77%	25.54%
Mean	32	13.3	10.2	6.35%	5.05%	1.27%	0.32%	18.40%

[a] No data. The lower portion of the table is calculated the same as Table 3-1. CVs are calculated for each individual experiment. The CVs of standard deviation for Experiments 827 and 903 are 7.03% and 0.94%, respectively.

CHAPTER 4
DISCUSSION

The CATS was designed as an ensemble to deliver a constant challenge of aerosolized pathogens through a test filter to a panel of mice, who serve as biological indicators of net viable penetration through two categories of test filters. The ensemble comprises a Collison nebulizer, a particle charge neutralizer, a filter holder and filter, and an animal exposure apparatus. Located throughout the system are ports from which the aerosol can be sampled with particle sizers and impingers. Pressure gauges and RH and temperature sensors are included to measure environmental conditions. The hypothesis tested in this work is that the challenge delivered to the animals is consistent. Variation in the challenge could arise from variations in the PSD of the upstream challenge, the airborne viable concentration, and the PRE or VRE of the filter. The criteria to confirm the hypothesis are that for each individual experiment the TPC, CMD, and GSD of the PSD and the airborne viable concentration have CVs below 20%.

Flow Rate, Relative Humidity, and Temperature Consistency

Precedents show that variations in flow rate, RH, and temperature affect both the PSD and the airborne viable concentration. The flow rate, RH, and temperature data show that the flow rate and temperature of the CATS remain within a range of 1% and RH is consistent within a range of 5%. This compares well with the literature: Bonnet et al.[78] maintained RH within ±5% in their system. Because the flow rate, RH, and temperature are consistent, they are not significant sources of variation.

Correlation of Sampling Ports

To prove that samples taken at different sampling ports on the CATS are equivalent, aerosols of 250-nm and 1-µm beads were created and the measurements at

different ports were compared. The deviations from the mean of particle readings between ports on the CATS are within ±10% of the mean with aerosolized 250-nm beads and the deviations with 1 µm beads are much lower. These measurements were performed with different instruments (SMPS for 250-nm beads, APS for 1-µm beads) and the difference in consistency may be a difference between the repeatability of readings on each instrument. The particle readings in this work did not decrease further along the flow path, suggesting minimal particle loss occurs in the CATS. The largest of these deviations is smaller than that measured on the system built by Oldham et al.,[81] although Oldham et al. were studying an animal exposure chamber much larger than the CATS. Measurements taken at different sampling ports on the CATS can be compared to each other with confidence.

Bioaerosol Consistency Trials

Particle Size Distribution

To measure variation in the PSD inside the CATS, measurements were taken with APS and SMPS particle sizers. The low CVs in Tables 3-1 and 3-3 for the statistical upstream quantities of the PSDs are all less than 10%, often very much less. The statistical properties of the PSDs showed no discernible trend upward or downward in time during the observation period. Previous work in which qualities of particle size distributions were measured can be compared. Raabe et al.[77] created a uranine aerosol to validate his animal exposure system, collected it on filters, and measured the change in filter weight. Their data can be transformed to give data points of mass concentration for individual time steps, and CVs can be calculated from that transformed data. The CVs for his two experiments are around 27%. Rihn et al.[76] measured mass concentration of aerosolized asbestos fibers within their system. From a single

experiment measuring mass collected on filters, they reported a CV for their mass concentration of 15%. Bonnet et al.[78] reported total mass concentrations of particles from fumes of bitumen collected on filters; among their three experiments, CVs ranged from 17% to 32%. Nadithe et al.[79] used an Aerosizer particle sizer to measure an aerosol of radiolabeled human serum albumin. They measured a CV for mass median aerodynamic diameter near 13% and a CV for GSD of 4%. Based on the CVs measured in this work, the PSD within the CATS was generally more consistent than that of other systems in the literature, and varied only slightly during the periods of observation.

The literature shows that the method used to measure the PSD can introduce a great deal of error into the measurement, and that some methods are more consistent than others. Particle sizers are more consistent than other methods, although particle sizers are significantly more expensive and require more upkeep than filters or cascade impactors. When particle sizers are available, their use is a good way to obtain consistent measurements of the bioaerosol's PSD.

The Collison is also a more consistent method of creating an aerosol than others, although the Collison cannot accommodate the smoke particles examined by Bonnet et al.[78] or fibers as studied by Rihn et al.[76] Since the CATS will only be used with bioaerosols, the Collison is a good choice to create a consistent bioaerosol challenge.

Viability

MS2

Experiments were performed with aerosolized MS2 coli phage to determine the consistency of a viral challenge. The PRE graph shown in Figure 3-2 suggests that viable particles were capable of penetrating. MS2 was collected in impingers upstream and downstream of a T-5000 medium and assayed, but all MS2 plates showed

contamination or were otherwise unusable, so viability data were not measured. Inexperience with the plating method likely contributed to lack of success producing viability data with MS2. As well, because no biological safety cabinet was available, the MS2 plating was performed on an open bench. While, for a BSL-1 organism, plating on the bench poses no hazard of infection, it increases the contamination risk from ambient air.

Since MS2 is a small particle it is dwarfed by the particles produced from the dissolved solids, and any evidence of MS2 in the particle size distribution is obscured by the dissolved solids mode. The MS2 particles are invisible in the PSDs, which, in the absence of viability data, are the only data collected. There is little difference between the experiments performed with MS2 and an equivalent set of experiments done with an inert challenge. However, given the success of other researchers in the literature in producing MS2 aerosols over durations similar to the length of these experiments, aerosolization of viable MS2 should be achievable in this system. Eventually MS2 work was halted and this work moved on to moved on to the next organism. Measurements of viability were not achieved for MS2 in this work, but its properties should not differ from the literature.

B. atrophaeus

Work with *B. atrophaeus* was begun after MS2 proved problematic. Experiments were performed with aerosolized *B. atrophaeus* to determine the consistency of the viability of a bacterial challenge. *B. atrophaeus* was collected in impingers and successfully assayed. Out of seven experiments performed, five experiments had usable plates. No viable penetration of *B. atrophaeus* through any filter was observed. A brief example calculation shows why the PREs of the filters used were too large to

observe viable penetration. If a nebulization liquid consisting of once-diluted stock (at a titer of 10^7 CFU/mL) were sprayed, then calculating based on the VSF for the entire system, an aerosol of 7.8×10^6 CFU/m^3 would be measured upstream of the filter. Using Equation 2-2, and assuming that the minimum number of colonies counted from a plate to have reliable data is N = 30, that the sampled aerosol flow rate is Q_a = 2.5 L/min, that the initial plates are counted (n = 0), and that everything else is the same as for the other experiments, the minimal detection limit of the impingers is about 5×10^4 CFU/m^3. Plugging a minimal downstream concentration of 5×10^4 CFU/m^3 and a maximal upstream concentration of 7.8×10^6 CFU/m^3 into Equation 1-1 gives a VRE of 99.4%. Therefore one should be able to detect aerosol with an impinger downstream of a filter with 99.4% VRE or lower, provided a large challenge concentration on the order of 10^7 CFU/m^3. PREs for all of the filters tested were all much higher than 99.4% in the size range of *B. atrophaeus*. Therefore, the penetration of *B. atrophaeus* can only be measured on a less-efficient filter; alternately, a smaller microorganism could be used.

Out of the five experiments, the largest CV for upstream airborne viable concentration in a single *B. atrophaeus* experiment was 26%, and another CV lay slightly outside the goal of 20%. The variability in viability is worse than observed by Henderson,[74] who was also using a *Bacillus* spore and measured a worst-case CV of 10.4%. The data lie outside the criteria to validate the hypothesis, but in retrospect that criterion was overly ambitious and not necessary for validating the CATS. The absence of a clear trend suggests that the few high CVs for viability are because of experimental noise rather than systematic decrease in viability. Inexperience with microbiological methods also likely contributed to the variability observed in the bacterial spore tests.

The airborne viable concentration is steady enough for use in an animal exposure, and the Collison is suitable for creating that challenge.

The VSF of the CATS estimated at the Collison nozzle for *B. atrophaeus* (2×10^{-6}) is comparable to the VSFs measured by Henderson[74] for *B. subtilis* at the end of his spray tube (3.5×10^{-6} to 4.1×10^{-6}), suggesting reasonably low loss due to the nebulization method. The regression used to determine the VSF has an R^2 higher than 90%, suggesting that the challenge atmosphere is fairly repeatable as well. Again, the Collison is a suitable method of creating a bacterial bioaerosol for an animal exposure.

With *B. atrophaeus*, the TPC or TPC above 0.8 µm did not show a correlation with the post-experiment Collison titer or the airborne viable concentration. The lack of correlation is unexpected: if there are more microorganisms, there should be more particles, especially at the size of that microorganism. The lack of correlation may be because the particles that make up the TPC above 0.8 µm are not necessarily viable. As shown in Table 3-2, in Experiment 901 and after, nominal titers were an order of magnitude smaller than the titer measured post-experiment. Data from this work show the aerosol does not significantly decrease in viability over an experiment, so the difference is not due to losses in the Collison. Experiment 901 and the ones following used a different lot of *B. atrophaeus* stock than the ones preceding. Stocks were titered by the laboratory staff where this work was performed, but something may have caused loss of viability between when it was first titered and when it was used. The lack of correlation is an inconsistency in laboratory methods rather than a flaw in the CATS.

Filter Physical Removal Efficiency

Unfortunately, no viable penetration was measured through the filters. Filter media tried in this work were either too efficient to measure penetration or achieved no

removal in the range of interest. For the later animal experiment, media with a low PRE (near 97% at a size of ~500 nm) has been specially manufactured by Safe Life, and a smaller microorganism (H1N1 Influenza A, with a particle size on the order of 100 nm) that can be nebulized at higher titers will be used. Not enough pieces of the special-order media were available for it to be used in this work as well as the animal trials.

Because penetration was not measured, and the downstream PSD was not consistently measured before and after the exposure period, the only observable parameter of the filter was its Δp, which did not observably change over the course of any experiment. The filter media used in this work were electrets, composed of polypropylene. Barret and Rousseau[30] showed that the behavior of electret polypropylene filters varies widely depending on how the fibers of the media were made, and that some lose PRE without showing a change in Δp. However, Barret and Rousseau were using NaCl and dioctyl phthalate aerosols specifically intended to reduce the PRE of electrets. Dioctyl phthalate is a strong plasticizer. It and other plasticizers do not appear in a bioaerosol tests. While salt may appear in a microbial stock, Barret and Rousseau were also using a challenge of 15 mg/m^3 of NaCl particles – a far larger mass concentration than encountered in a bioaerosol test – at similar face velocity for nearly 3 hours. In Experiment 20090910, for instance, the total mass concentration of the challenge was only about 0.6 mg/m^3, and very little of that mass was salt. The bioaerosol challenges that the CATS is used with likely do not have quite the capacity to reduce PRE that Barret and Rousseau's aerosol challenges did, and no previous studies on PSTI electret media have showed such a reduction in PRE. Bioaerosols likely do not reduce the PRE of electret filters significantly.

Measurements of PSD were taken downstream before and after loading in only Experiment 20090910 on the IPA-treated 1860S medium, which had had its electric charge removed by the IPA treatment. Since that filter was no longer an electret, it should not be expected to have the reduction in PRE with loading that some electrets have, and the difference between upstream and downstream measurements in Figure 3-4 likely is due to experimental error. There is no reason to believe that the PRE changed over the course of the experiments performed in this work.

It should be noted that all of the test cases in this work used a nebulization liquid that had relatively low concentrations of dissolved solids, thus causing a lower loading on the filter. The microorganism initially chosen for the animal exposure was *Francisella tularensis*, which is not as stable in water and requires a larger amount of dissolved protein content in its nebulization liquid. Section 3.2 of Heimbuch et al.[100] details the preliminary work that determined that *F. tularensis* was not an appropriate challenge for the animal study. When *F. tularensis* was aerosolized through the test filter, in a setup similar to the CATS, the filter medium was rapidly loaded by dissolved solids and its PRE approached 100% quickly. That the PRE of the fabric increased instead of decreased under heavy bioaerosol loading gives credence to the idea that bioaerosols do not have the capacity to significantly reduce the PRE of Safe Life's filters.

Extrapolating to the Delivered Dose

That viable penetration by a micrometer-sized bacterium was not measured does not invalidate the performance of the CATS. Data in Tables 3-1 and 3-3 show that the upstream challenge is consistent for the duration of the experiments. There is no reason to believe that the filters in this work had any change in their PRE, so one can predict

that the downstream challenge would also be consistent in tests with filters with lower PREs and not contribute variability to the dose.

As said earlier, simply measuring the actual dose received by the animal is, when possible, the best metric for validation. Kaur et al.[80] state that in their system, in which mice were exposed to aerosolized dry powders of anti-tuberculosis drugs, the dose received had a CV of 13.5% or lower, and the dose was accurate enough that no significant difference was observed between mice dosed intravenously and by the aerosol route. Raabe et al.[77] exposed mice to ^{137}Cs aerosol particles and measured a CV for the lung burden among 80 Syrian hamsters of 25%. While an animal exposure was not performed in this work, it can be reasoned that the variability of the dose is driven by the most-variable component of Equation 1-3, which in this work is the airborne viable concentration. The variability of airborne viable concentration in this work is higher than Kaur et al.'s variation in dose and slightly higher than Raabe et al.'s. Again, what part of the variability in the viable counts in this work is not an artifact of the viability measurement method is unclear. The CVs measured for viable counts are low enough that a dose with that CV or slightly higher is acceptable in an animal exposure.

The data support a conclusion that the CATS satisfied the key conditions to maintain a consistent challenge: PSDs remained acceptably constant, airborne viable concentration was fairly consistent, and PRE can be reasoned to have not changed discriminably. One can conclude that CATS is capable of producing an acceptably consistent challenge for dosing animals in an exposure trial. The CATS cannot accommodate every conceivable combination of organism and filter, but its operating envelope is wide enough to enable the PSTI-filter animal exposure trials.

CHAPTER 5
CONCLUSION

To enable an animal inhalation study that will evaluate the effect of an antimicrobial filter on the infectivity of bioaerosols, an experimental system to expose rodents to aerosols that have passed through a filter was designed and built, and its mechanical performance was validated. Aerosol challenges of MS2 coli phage virus and the bacteria *Bacillus atrophaeus* were created and flowed through the system, and thence through coupons of filter media cut from commercially available FFRs. However, viability of MS2 was not measured because of assay problems, and penetration by *B. atrophaeus* was too small to quantify.

Two commercially available FFRs, the T-5000 and 1860S, have very large PREs near 1 µm, too large for use in an animal test using bacteria. No significant viable penetration was observed in challenge experiments because of these filters' high PRE. However, the Δp across the filters remained constant, and no sign was found that PRE changed over the course of the experiments. The upstream PSD was very consistent during these tests, with CVs all less than 10%. The upstream viable airborne concentration of airborne *B. atrophaeus* was suitably consistent, with CVs of less than 26%, comparable to the literature. This maximum observed CV is larger than the criteria to validate the hypothesis, but that goal was likely too ambitious.

From the data in this work, and reasoning based on the literature, one can conclude that the downstream PSD and viable airborne concentration remain steady for long enough to accurately deliver the challenges needed to perform the animal exposure trials. The CATS can produce a bioaerosol challenge that is sufficiently uniform to support statistically reliable animal infectivity testing. The CATS provides a

design for an animal exposure system incorporating aerosol filtration, a capability previously unreported in the literature.

APPENDIX A
OPERATING SEQUENCES

Leak Check

Step 1. Metal stubs of ½-inch diameter were placed into ports and the fittings were tightened. All ports on the mouse tree were plugged.

Step 2. Valve 1 was opened until pressure in the system had reached approximately 0.25 psig. The exact value was not important, but if the pressure was too high, the plugs in the mouse tree would begin to creep out, affecting the reading. The charge neutralizer has a stated maximum pressure of 5 psig.

Step 3. The pressure readings were observed. If, after one hour or so, the pressure was still what it was initially, the leak check was successful. If not, seals and fittings were checked.

Pre-Nebulization Preparations

Step 1. The system was depressurized and air bled from the humidification loop. Air creeps into the humidifying loop over the course of an experiment because of the pressure difference. The air needs to be purged and the tube allowed to completely soak before pressurizing it again. This step was done at least an hour before nebulization, but could be done at any time after the previous experiment.

1. The plug at the end of the bleed stem was opened. Any water in the line was let to leak out into a small glass. If any air was in the line, the flow would stop.
2. A pipetter and a length of tubing were used to draw flow from the bleed stem until siphon pressure caused the water to flow freely.
3. The plug at the end of the bleed stem was closed.

Step 2. The filter was inserted into the filter holder.

1. The two halves of the filter holder were separated by unscrewing the screws using a ball-tipped hex driver (or other hex driver or an Allen wrench).

2. The upstream section of the CATS was pulled back, and the downstream section of the filter holder was rotated outwards so that it could be accessed.

3. The filter was inserted. For 47-mm samples, it was inserted so that the mesh was downstream, the O-ring upstream, and the upstream side of the filter material facing the correct direction. From the vantage of looking into the filter holder this appeared as the O-ring in front and the mesh behind.

4. The filter holder was closed. The downstream and upstream sections were aligned and brought together, and then screwed together with the hex driver. The filter holder was then examined visually to make sure the two halves of the filter holder were level.

Step 3. Airflow through the system was begun by turning on the main air to the system Valve A. This air was flowed through the filter to blow off the initial iodine bloom. The system was sampled before the filter (at 1) with a particle sizer to confirm that the CATS was clear of particles. The pressure gauges were checked for correct readings.

Aerosol Consistency Trials

Step 1. The correct starting valve configuration was confirmed as so:

- Off: Valves B, C, D, 1, 2, 4, 5a, 5b.
- On: Valves A, 3.

Step 2. The cap was removed and a filled nebulizer was attached. The ½-inch Swagelok® nut attaching the nebulizer to the CATS was tightened. The nebulizer's pressurized air line was connected using its Ultra-Torr fitting.

Step 3. The Collison was turned on at Valve D. The impinger dilution air at Valve C was also turned on, as the change in pressure from the air going to the impingers can affect the main flow rate by ±0.2 L/min. Valve A was adjusted to produce a flow such that the face velocity through the media was correct. For the tests with PSTI media, this velocity is specified as 7.08 cm/s. For a 47-mm sample of which 40 mm experiences flow, the corresponding flow rate is 5.3 L/min. Also, the flow rate of the impinger dilution air was adjusted to an appropriate level for the test (10 to 11 L/min).

Step 4. The flow was allowed to equilibrate. A period of 15 minutes is standard and appeared to be sufficient.

Step 5. After equilibration, upstream (Port 1) and downstream (Port 2) measurements were made with the particle sizer.

Step 6. The first impinger sample was begun at the downstream, as follows:

1. The dilution air (Valve C) was turned off.
2. The impinger sampling hose was connected to Port 2. Valve 2 was not opened at this step.
3. The impinger was connected to Port 5b and the Ultra-Torr fitting was tightened. Valve 5b was opened.
4. The dilution air (Valve C) was turned on.
5. The vacuum pump was connected to the impinger.
6. Valve 2 was opened, beginning the sampling. A 5-minute timer was started.

Step 7. Sampling with the impingers was performed for a period of 5 minutes per impinger, alternating upstream and downstream. To reduce contamination and backflow, the following procedure was used when switching. For simplicity, this sequence is couched as switching from downstream to upstream: for the other way, just swap Valves and Ports 1 and 2, and 5a and 5b. Particle sizer measurements were taken at points during this step but did not require a special sequence to prevent contamination.

1. Valve 2 was closed.
2. The vacuum pump tube was removed from the impinger.
3. The dilution air (Valve C) was turned off.
4. The impinger sampling hose was disconnected from Port 2, and inserted into a HEPA capsule.
5. Valve 5b was closed.
6. The dilution air (Valve C) was briefly turned back on to purge the impinger sampling hose. A purge was performed for at least 15 seconds to remove remaining aerosol.
7. The dilution air (Valve C) was turned back off.
8. The impinger sampling hose was connected to port 2. Valve 2 was not opened.
9. The impinger was connected to Port 5b and the Ultra-Torr fitting was tightened. Valve 5b was opened.

10. The dilution air (Valve C) was turned on.
11. The vacuum pump was connected to the impinger.
12. Valve 2 was opened. The timer was started.

Step 8. Once impinger sampling was finished, upstream (Port 1) and sometimes downstream (Port 2) measurements were made with the particle sizer. (This step was not always performed.)

Step 9. Collison flow was turned off, the nebulizer removed and the cap replaced. Air was flowed to purge the system.

Step 10. If sampling a biological challenge, the impinger aliquot and remaining Collison liquid were assayed.

APPENDIX B
FLOW RATE, TEMPERATURE, AND RELATIVE HUMIDITY DATA

Temperature, relative humidity, and flow rate data measured on the CATS over a period of more than an hour are presented in Table B-1. Data begin from the absolute start of beginning flow: there is no equilibration period. Note that the relative humidity starts off low, illustrating the importance of letting the system equilibrate before beginning measurements. The low initial RH measurement is excluded from the mean. Data after 65 minutes are taken to show the effect of toggling the flow to the impingers, which causes a deviation in flow rate of about 2%.

Table B-1. Temperature, RH, and flow consistency data

				% deviation from mean		
Time (min)	T (°C)	RH	Flow (L/min)	T	RH	Flow rate
0	20.7	44.7%	5.28	0.47%		-0.50%
5	20.7	71.5%	5.31	0.47%	1.70%	0.18%
15	20.6	72.4%	5.32	0.22%	3.01%	0.33%
20	20.6	71.5%	5.31	0.13%	1.70%	0.16%
25	20.6	70.8%	5.31	0.08%	0.76%	0.12%
30	20.6	70.4%	5.30	0.03%	0.23%	0.05%
35	20.6	70.1%	5.30	-0.07%	-0.25%	-0.03%
40	20.6	69.9%	5.30	-0.12%	-0.59%	-0.05%
45	20.6	69.8%	5.30	-0.16%	-0.72%	-0.03%
50	20.5	69.6%	5.30	-0.21%	-1.02%	-0.07%
55	20.5	69.2%	5.30	-0.21%	-1.50%	-0.03%
60	20.5	69.1%	5.30	-0.31%	-1.63%	-0.07%
65	20.5	69.1%	5.30	-0.31%	-1.67%	-0.07%
Mean of above	20.6	68.3%	5.30			
70	20.6	68.1%	5.43	-0.16%	-3.10%	2.35%
75	20.5	68.5%	5.42	-0.21%	-2.59%	2.20%
80	20.5	68.9%	5.41	-0.21%	-2.02%	1.95%
85	20.5	69.0%	5.40	-0.21%	-1.80%	1.86%
90	20.5	68.9%	5.40	-0.26%	-1.93%	1.86%

APPENDIX C
PORT CORRELATION DATA

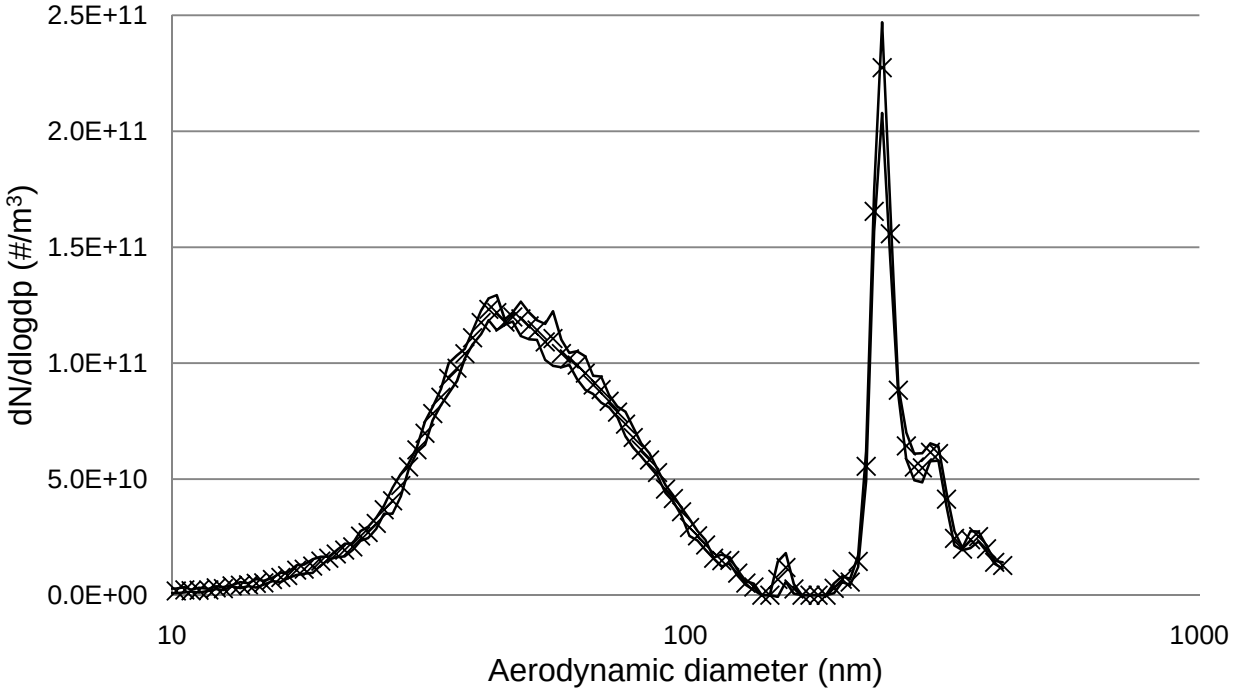

Figure C-1. Representative PSD from nebulizing 250-nm beads. Taken from the first set of samples at Port 1. Lines are 95% confidence intervals for each diameter. The peak consistently occurred at 241.4 nm and was fairly sharp.

Table C-1. Readings of particle concentration at ports on Controlled Aerosol Test System (CATS) while nebulizing 250-nm beads

Sampling port	Concentration, 232.9 to 250.3 nm (10^6 #/m^3)			Sample mean	Deviation from overall mean
Port 4	9712	9833	9739	9761	3.16%
Port 1	8666	9061	9220	8982	-5.08%
Port 2	9384	9228	9295	9302	-1.69%
Port 4	9123	9429	9411	9321	-1.50%
Port 1	8382	8402	8935	8573	-9.40%
Port 2	9205	9418	9288	9303	-1.68%
Port 4	9903	10073	10129	10035	6.05%
Port 1	9351	9371	9432	9385	-0.82%
Port 5a	9836	9575	9570	9660	2.09%
Port 5b	9573	9668	9398	9546	0.89%
Mouse tree, quad 1	9663	9505	9664	9611	1.57%
Mouse tree, quad 2	9009	9975	10052	9679	2.28%
Mouse tree, quad 3	9529	9949	10050	9843	4.02%
Mouse tree, quad 4	9138	9740	9540	9473	0.11%
Overall mean				9463	(Max-min)
Minimum				8573	/mean
Maximum				10035	15.45%

The entire experiment lasted four hours after aerosol equilibration.

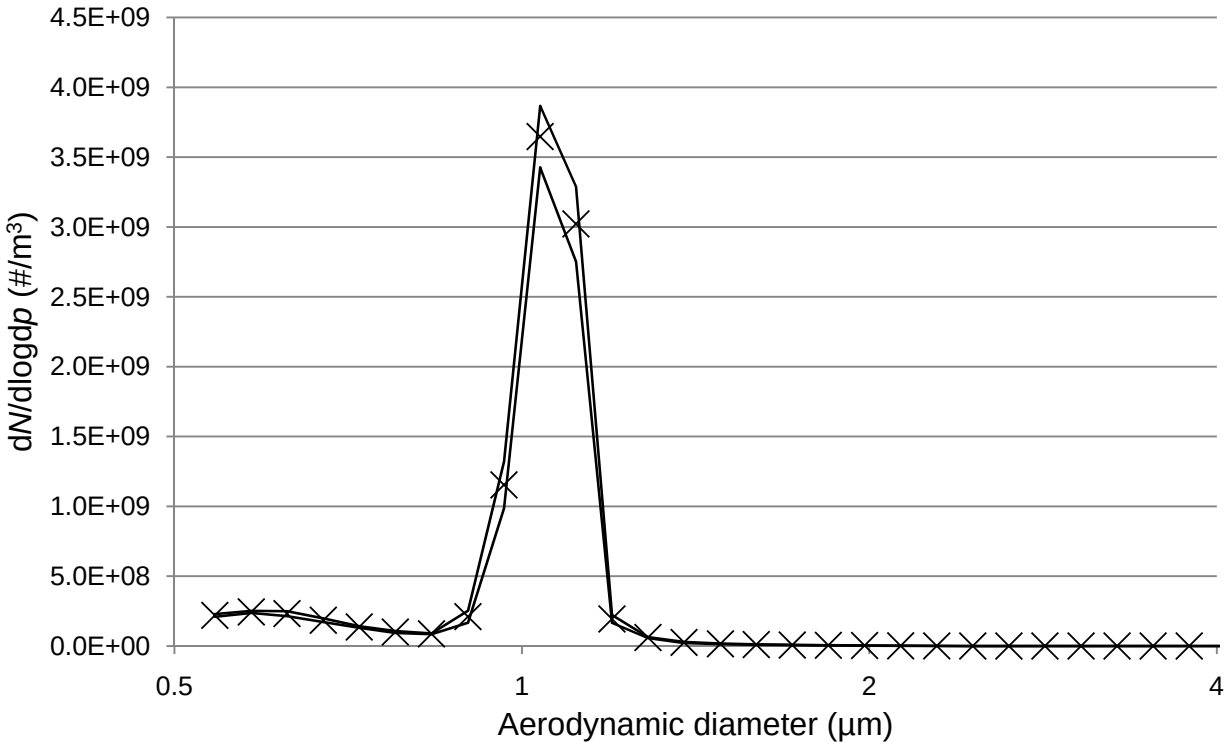

Figure C-2. Representative PSDs from nebulizing 1-µm beads. Data is based on samples taken at quadrants of the mouse tree. Lines are 95% confidence intervals. A broad, slightly unsymmetrical peak consistently occurred at 1.037 µm.

Table C-2. Readings of particle concentration at ports on CATS while nebulizing 1-µm beads

Port	Concentration, 0.965 to 1.114 µm (10^6 #/m^3)	Deviation from mean
Port 4	246	-0.68%
Port 2	246	-0.39%
Port 1	249	0.60%
Port 5b	251	1.68%
Port 5a	249	0.63%
Quad 1	247	-0.14%
Quad 2	245	-0.83%
Quad 3	242	-1.95%
Quad 4	242	-2.11%
Quad 4	246	-0.53%
Port 2	250	1.04%
Port 1	254	2.68%
Overall mean	247	
Minimum	242	(Max-min)/mean:
Maximum	254	4.79%

This correlation took only 30 minutes after aerosol equilibration.

APPENDIX D
BIOAEROSOL CONSISTENCY RAW DATA

In the tables of PSDs, elapsed time is the time at the end of the sampling period minus the time the run was started; TPC, CMD, and GSD are the moments calculated by the particle sizer software; mean is the mean across each row; St. dev. is the standard deviation across each row; and CV is the ratio of st. dev. to mean. In the tables for the PSD of *B. atrophaeus*, TPC >0.8 is the concentration of particles with aerodynamic diameter larger than 0.8 µm. Note that the magnitude of TPC and TPC >0.8 varies from table to table for *B. atrophaeus*.

In the tables of viability, the Dilution column indicates n in the dilution ratio 10^{-n}. Because the plating method adds another 1:10 dilution, the readings from the plates were multiplied by 10, which is the reason all the raw counts end in zero. The collected aerosol flow rate is denoted Q_a. Nominal titer is the titer of the nebulization liquid calculated from its dilution ratios and the titer of the undiluted stock. Except where specified, the volume of nebulizer liquid was 30 mL. The lot number is an in-laboratory identifier for each batch of freezer stock of *B. atrophaeus*. All MS2 plates were contaminated or otherwise unusable. No viability data are recorded for *B. atrophaeus* experiments 827, 903, and 909 because the plates were contaminated: where viability was not measured, the nominal titer appears in the notes to the PSD table. NR indicates data not recorded.

MS2

Table D-1. PSD data for Experiment 724 (MS2)

Elapsed time	19:17	21:36	32:04	34:23	45:04	47:23	Mean	St. dev.	CV
TPC (10^{12} #/m^3)	2.74	2.69	2.50	2.51	2.68	2.80	2.65	0.124	4.66%
CMD (nm)	79.8	81.4	81.5	81.9	80.9	82.8	81.4	1.00	1.23%
GSD	1.70	1.70	1.70	1.70	1.71	1.70	1.70	0.00327	0.19%

Mean T: NR. Mean RH: 65%. Δp: 0.8 in H_2O. Nominal titer: NR.

Table D-2. PSD data for Experiment 728 (MS2)

Elapsed time	19:42	22:01	33:50	36:08	46:29	48:47	Mean	St. dev.	CV
TPC (10^{12} #/m^3)	4.86	4.64	4.57	4.36	4.20	4.40	4.51	0.235	5.21%
CMD (nm)	71.5	71.1	76.2	75.4	75.3	75.8	74.2	2.30	3.09%
GSD	1.71	1.71	1.68	1.68	1.68	1.68	1.69	0.0172	1.02%

Mean T: 23 °C. Mean RH: 61%. Δ*p*: 0.84 in H_2O. Nominal titer: 10^{12} PFU/mL.

Table D-3. PSD data for Experiment 730 (MS2)

Elapsed time	17:54	20:13	30:53	33:11	43:35	45:54	Mean	St. dev.	CV
TPC (10^{12} #/m^3)	4.34	4.28	4.00	4.04	4.21	4.10	4.16	0.138	3.31%
CMD (nm)	74.8	75.0	75.2	76.2	76.0	77.2	75.7	0.907	1.20%
GSD	1.68	1.68	1.68	1.69	1.68	1.68	1.68	0.00360	0.21%

Mean T: 22 °C. Mean RH: 64%. Δ*p*: NR. Nominal titer: 10^{11} PFU/mL.

Table D-4. PSD data for Experiment 811 (MS2)

Elapsed time	16:54	19:12	30:05	32:24	43:40	45:59	Mean	St. dev.	CV
TPC (10^{12} #/m^3)	0.500	0.544	0.514	0.513	0.503	0.503	0.513	0.0162	3.16%
CMD (nm)	74.1	73.9	75.5	75.7	76.9	77.6	75.6	1.46	1.93%
GSD	1.72	1.70	1.72	1.72	1.71	1.72	1.72	0.00960	0.56%

Mean T: 27 °C. Mean RH: 55%. Δ*p*: NR. Nominal titer: NR.

Table D-5. PSD data for Experiment 812 (MS2)

Elapsed time	19:05	21:23	32:34	34:52	45:06	47:24	Mean	St. dev.	CV
TPC (10^{12} #/m^3)	4.91	4.71	4.53	4.46	4.22	4.22	4.51	0.270	6.00%
CMD (nm)	75.9	76.2	76.7	76.8	76.7	76.9	76.5	0.374	0.49%
GSD	1.70	1.70	1.70	1.70	1.70	1.70	1.70	0.00144	0.08%

Mean T: 27 °C. Mean RH: 53%. Δ*p*: NR. Nominal titer: NR.

Table D-6. PSD data for Experiment 813 (MS2)

Elapsed time	18:29	20:47	31:18	33:36	43:28	45:46	Mean	St. dev.	CV
TPC (10^{12} #/m^3)	4.30	4.28	4.19	4.22	3.88	3.81	4.11	0.212	5.15%
CMD (nm)	77.9	78.1	78.3	78.2	77.6	77.7	78.0	0.286	0.37%
GSD	1.70	1.70	1.70	1.69	1.70	1.71	1.70	0.00493	0.29%

Mean T: 26 °C. Mean RH: 56%. Δ*p*: NR. Nominal titer: NR.

B. atrophaeus

Table D-7. PSD data for Experiment 819 (*B. atrophaeus*)

Elapsed time	14:54	22:27	30:12	37:37	45:42	53:22	60:39	Mean	St. dev.	CV
TPC (10^{7} #/m^3)	12.2	11.8	11.4	12.0	12.9	12.9	13.1	12.3	0.642	5.21%
TPC >0.8 (10^{7} #/m^3)	7.69	7.34	6.92	7.24	7.40	7.35	7.36	7.33	0.229	3.12%
CMD (µm)	1.09	1.08	1.07	1.08	1.07	1.07	1.06	1.08	0.00742	0.69%
GSD	1.32	1.32	1.32	1.32	1.33	1.33	1.33	1.33	0.00560	0.42%

Mean T: 24 °C. Mean RH: 58%. Δ*p*: 0.9 in H_2O.

Table D-8. Viability data for Experiment 819 (*B. atrophaeus*)

Source	Dilution	Plate counts			Mean	Concentration
Nebulizer	4	1460	1440	1320	1407	
Liquid	5	280	300	210	263	2.63×10^{7} CFU/mL
Upstream	1	1130	1200	840	1057	
sample 1	2	180	130	170	160	2.56×10^{7} CFU/m^3
Upstream	1	840	1050	1050	980	
sample 2	2	160	140	190	163	2.61×10^{7} CFU/m^3
Upstream	1	990	930	940	953	
sample 3	2	130	150	60	113	1.81×10^{7} CFU/m^3
Mean of upstream						2.33×10^{7} CFU/m^3
Standard deviation						4.47×10^{6} CFU/m^3
CV						19.21%

Q_a = 2.5 L/min. Nominal titer: 10^7 CFU/mL, from Lot 07-08-29.

Table D-9. PSD data for Experiment 820 (*B. atrophaeus*)

Elapsed time	15:09	16:11	23:36	32:34	39:46	47:56	55:02	66:05	Mean	St. dev.	CV
TPC (10^7 #/m^3)	5.31	5.40	5.70	5.80	6.08	6.15	6.35	6.54	5.92	0.439	7.42%
TPC >0.8 (10^7 #/m^3)	4.56	4.60	4.84	4.87	5.04	5.08	5.17	5.28	4.93	0.259	5.26%
CMD (µm)	1.11	1.10	1.10	1.09	1.09	1.09	1.09	1.09	1.10	0.00652	0.60%
GSD	1.20	1.21	1.21	1.21	1.22	1.22	1.22	1.22	1.21	0.00697	0.58%

Mean T: 24 °C. Mean RH: 57%. Δ*p*: NR.

Table D-10. Viability data for Experiment 820 (*B. atrophaeus*)

Source	Dilution	Plate counts			Mean	Concentration
Nebulizer	4	980	1100	1020	1033	
liquid	5	90	160	170	140	1.40×10^{7} CFU/mL
Upstream	1	580	600	520	567	9.07×10^{6} CFU/m^3
sample 1	2	90	110	110	103	
Upstream	1	350	420	470	413	6.61×10^{6} CFU/m^3
sample 2	2	30	50	180	87	
Upstream	1	370	400	310	360	5.76×10^{6} CFU/m^3
sample 3	2	50	40	80	57	
Mean of upstream						7.15×10^{6} CFU/m^3
Standard deviation						1.72×10^{6} CFU/m^3
CV						24.02%

Q_a = 2.5 L/min. Nominal titer: 10^7 CFU/mL, from Lot 07-08-29.

Table D-11. PSD data for Experiment 827 (*B. atrophaeus*)

Elapsed time	13:02	22:12	29:22	36:32	43:51	51:24	58:30	Mean	St. dev.	CV
TPC (10^7 #/m^3)	2.55	2.92	2.92	3.19	3.40	3.19	3.38	3.08	0.301	9.77%
TPC >0.8 (10^7 #/m^3)	0.743	0.721	0.664	0.658	0.653	0.582	0.574	0.656	0.0634	9.66%
CMD (µm)	0.789	0.727	0.711	0.691	0.681	0.673	0.660	0.705	0.0435	6.18%
GSD	1.36	1.36	1.36	1.35	1.35	1.35	1.34	1.35	0.0104	0.77%

Mean T: 23 °C. Mean RH: 63%. Δ*p*: 0.2 in H_2O. Nominal titer: 10^7 CFU/mL, from Lot 07-08-29.

Table D-12. PSD data for Experiment 901 (*B. atrophaeus*)

Elapsed time	01:49	08:45	15:38	22:51	30:20	37:39	44:48	Mean	St. dev.	CV
TPC (10^8 #/m^3)	2.63	2.36	2.51	2.34	2.46	2.77	2.74	2.54	0.173	6.81%
TPC >0.8 (10^8 #/m^3)	1.20	1.14	1.22	1.18	1.24	1.25	1.26	1.21	0.0441	3.64%
CMD (µm)	0.948	0.957	0.957	0.961	0.958	0.943	0.948	0.953	0.00670	0.70%
GSD	1.33	1.33	1.33	1.33	1.33	1.33	1.33	1.33	0.00203	0.15%

Mean T: 23 °C. Mean RH: 50%. Δ*p*: 0.26 in H_2O. On this experiment, the equilibration time appears to be highly abbreviated, although this may just be a mistake in noting the time. The short equilibration time did not seem to affect the results.

Table D-13. Viability data for Experiment 901 (*B. atrophaeus*)

Source	Dilution	Plate counts			Mean	Concentration
Nebulizer	3	1430	1420	1640	1497	
liquid	4	360	280	200	280	2.80×10^6 CFU/mL
	5	10	20	40	23	
Upstream	0	620	560	730	637	
sample 1	1	240	200	250	230	3.68×10^6 CFU/m^3
Upstream	0	860	720	630	737	
sample 2	1	210	140	160	170	2.72×10^6 CFU/m^3
Upstream	0	750	720	680	717	
sample 3	1	160	180	170	170	2.72×10^6 CFU/m^3
Mean of upstream						3.04×10^6 CFU/m^3
Standard deviation						5.54×10^5 CFU/m^3
CV						18.23%

Q_a = 2.5 L/min. Nominal titer: 1.6×10^7 CFU/mL, from Lot 09-09-01.

Table D-14. PSD data for Experiment 903 (*B. atrophaeus*)

Elapsed time	13:49	21:29	29:40	37:08	44:36	51:39	59:49	Mean	St. dev.	CV
TPC (10^8 #/m^3)	2.54	2.57	2.56	2.37	2.59	2.75	2.74	2.59	0.129	5.00%
TPC >0.8 (10^8 #/m^3)	1.15	1.18	1.19	1.11	1.24	1.25	1.25	1.20	0.0548	4.58%
CMD (µm)	0.944	0.944	0.946	0.940	0.945	0.940	0.936	0.942	0.00334	0.35%
GSD	1.34	1.34	1.34	1.34	1.34	1.34	1.34	1.34	0.00111	0.08%

Mean T: 23 °C. Mean RH: 48%. Δ*p*: 0.3 in H_2O. Nominal titer: 1.6×10^7 CFU/mL, from Lot 09-01-09.

Table D-15. PSD data for Experiment 908 (*B. atrophaeus*)

Elapsed time	15:59	23:57	31:17	38:26	45:29	52:15	59:18	Mean	St. dev.	CV
TPC (10^8 #/m^3)	5.22	5.15	5.08	5.18	5.19	5.53	5.60	5.28	0.203	3.84%
TPC >0.8 (10^8 #/m^3)	2.39	2.45	2.48	2.56	2.58	2.63	2.69	2.54	0.107	4.21%
CMD (µm)	0.948	0.951	0.953	0.951	0.948	0.943	0.943	0.948	0.00398	0.42%
GSD	1.35	1.34	1.34	1.34	1.34	1.34	1.34	1.34	0.00297	0.22%

Mean T: 22 °C. Mean RH: 47%. Δ*p*: 0.31 in H_2O.

Table D-16. Viability data for Experiment 908 (*B. atrophaeus*)

Source	Dilution	Plate counts			Mean	Concentration
Nebulizer	3	2510	2730	3160	2800	
liquid	4	520	670	480	557	5.57×10^{6} CFU/mL
Upstream	1	140	210	380	243	6.49×10^{6} CFU/m^3
sample 1	2	20	20	0	13	
Upstream	1	250	130	170	183	4.89×10^{6} CFU/m^3
sample 2	2	60	30	0	30	
Upstream	1	150	140	150	147	3.91×10^{6} CFU/m^3
sample 3	2	10	30	20	20	
Mean of upstream						5.10×10^{6} CFU/m^3
Standard deviation						1.30×10^{6} CFU/m^3
CV						25.54%

Q_a = 1.5 L/min. Nominal titer: 4×10^{7} CFU/mL in 18 mL, from Lot 09-09-01.

Table D-17. PSD data for Experiment 909 (*B. atrophaeus*)

Elapsed time	12:54	21:01	28:00	35:17	42:24	49:12	56:11	Mean	St. dev.	CV
TPC (10^9 #/m^3)	1.139	1.120	1.138	1.162	1.172	1.313	1.410	1.208	0.110	9.12%
TPC >0.8 (10^9 #/m^3)	0.561	0.548	0.560	0.576	0.587	0.596	0.634	0.580	0.0289	4.97%
CMD (µm)	0.960	0.956	0.955	0.954	0.955	0.939	0.938	0.951	0.00862	0.91%
GSD	1.34	1.34	1.34	1.34	1.34	1.34	1.34	1.34	0.00255	0.19%

Mean T: 23 °C. Mean RH: 47%. Δp: 0.25 in H_2O. Nominal titer: 8×10^{7} CFU/mL in 16 mL, from lot 09-09-01.

Table D-18. PSD data for Experiment 910 (*B. atrophaeus*)

Elapsed time	14:31	22:57	30:01	37:55	44:52	52:22	59:23	Mean	St. dev.	CV
TPC (10^9 #/m^3)	1.206	1.135	1.097	1.201	1.186	1.192	1.199	1.174	0.0414	3.52%
TPC >0.8 (10^9 #/m^3)	0.589	0.565	0.537	0.608	0.599	0.609	0.620	0.590	0.0291	4.94%
CMD (µm)	0.961	0.962	0.954	0.962	0.959	0.960	0.962	0.960	0.00291	0.30%
GSD	1.34	1.34	1.34	1.34	1.34	1.34	1.34	1.34	0.00191	0.14%

Mean T: 23 °C. Mean RH: 45%. Δp: 0.26 in H_2O.

Table D-19. Viability data for Experiment 910 (*B. atrophaeus*)

Source	Dilution	Plate counts			Mean	Concentration
Nebulizer	5	250	180	110	180	1.80×10^{7} CFU/mL
liquid	6	50	10	20	27	
Upstream	1	380	430	590	467	1.24×10^{7} CFU/m^3
sample 1	2	40	40	30	37	
	3	0	10	10	7	
Upstream	1	540	530	400	490	1.31×10^{7} CFU/m^3
sample 2	2	70	20	30	40	
	3	10	10	20	13	
Upstream	1	450	490	390	443	1.18×10^{7} CFU/m^3
sample 3	2	40	20	80	47	
	3	10	0	0	3	
Mean of upstream						1.24×10^{7} CFU/m^3
Standard deviation						6.22×10^{5} CFU/m^3
CV						5.00%

Q_a = 1.5 L/min. Nominal titer: 8×10^{7} CFU/mL in 16 mL, from Lot 09-09-01.

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BIOGRAPHICAL SKETCH

Brenton Ross Stone was born in 1984 to parents Suzanne and Michael Stone. He is a native of the central New York town of Dolgeville, and graduated from Dolgeville's James A. Green High School in June of 2002. He began attending the University of Buffalo – State University of New York in 2002 as a member of the University Honors Program and graduated summa cum laude in 2006 with the degree of Bachelor of Science in mathematics, concentrating in applied mathematics.

After receiving his undergraduate degree, Brenton moved to Panama City, FL to work with Applied Research Associates, Inc., a contractor that supplies laboratory and technical support to the Air Force Research Laboratory. In 2008 he began distance coursework through the University of Florida Department of Environmental Engineering Sciences, with tuition support from his employer. Brenton received the degree of Master of Science in environmental engineering sciences in May of 2010.